Amyloid Enhancing Factor Activity is Associated with Ubiquitin

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To my beloved wife, my parents, and all those

who provided the support for completing this work

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ABBREVIATIONS

AA: Amyloid A protein
ABC: avidin-biotin-peroxidase complex
ABPP: Amyloid ß-precursor protein
AD: Alzheimer's disease
AE: Endocrine amyloid protein
AEF: Amyloid enhancing factor
AF: Familial amyloid neuropathy amyloid
AH: Haemodialysis associated amyloid protein
AHC: Alveolar hydatid cyst
AHD: Alveolar hydatid disease
AL: Light chain amyloid protein
AP: Amyloid-P component
APF: ATP-dependent proteolysis
APAGE: Alkaline polyacrylamide gel electrophoressi
Arg: Arginine
ATP: adenosine triphosphate
BCG: Bacille Calmitte-Guerin
bFGF: basic fibroblast growth factor
BiP: heavy chain binding protein
BSA: bovine serum albumin
BU: bovine ubiquitin
C: complement fragment
cDNA: complementary deoxyribonucleic acid
d: dalton
DMSO: dimethyl sulphoxide
DS: Dawn's syndrome
EACA: ϵ-amino-n-caproic acid
EDTA: Ethylenediamine tetra acetic acid
EGF: epidermal growth factor
ELISA: Enzyme linked immunosorbent assay
EP: electroeluted peptide
FAEF: fibril associated amyloid enhancing factor
FAP: familial amyloid polyneuropathy
FCS: Fetal calf serum
FMF: Familial Mediteranean Fever
Gln: Glutamine
Glu: Glutamic acid
Gly: Glycine
GRP: glucose regulated protein
H2A: histone 2A
HBSS: Hank's Balanced Salt Solution
HCHWA-D: Hereditary cerebral hemorrhage of Dutch type
HDL: High density lipoprotein
HSP: heat shock protein
IA: Islet amyloid
IAPP: Islet amyloid polypeptide
IC: Immune complex
Ig: Immunoglobulin

IL-1: Interleukin-1
i.p: Intraperitoneal
i.v: Intravenous
Kd: Killo dalton
KPI: Kunitz type protease inhibitor
Leu: Leucine
LPS: Lipopolysaccharide
MAP: Microtubule associated protein
ME: Mercaptoethanol
Met: Methionine
mol. wt.: molecular weight
mRNA: messenger ribonucleic acid
NC: Nitrocellulose
NFT: Neurofibrillary tangle
NGF: nerve growth factor
NP: Neuritic plaque
PBS: Phosphate buffered saline
pCMB: parachloro mercuribenzoic acid
PEG: polyethylene glycol
PFA: Perifollicular area
PGE: Prostaglandin E
PHF: Paired helical fillaments
p.i.: post infection
pI: Isoelectric point
P_L: Lambda left promoter
PMN: Polymorphonuclear leucocytes
PMSF: Phenylmethyl sulphonyl fluoride
PNPP: para-nitro phenyl phosphate
PrP: Prion protein
PTH: Phenylthiohydantoin
RAA: rabbit anti-AA antibody
RABU: rabbit anti-bovine ubiquitin antibody
Rc: Recombinant
SAA: Serum amyloid A protein
SAP: Serum amyloid P component
SBTI: soybean trypsin inhibitor
s.c.: subcutaneous
SDAT: Senile dementia of Alzheimer type
SDS-PAGE: Sodium dodecyle sulphate polyacrylamide gel electrophoresis
Ser: Serine
TBS: Tris buffered saline
TGF: transforming growth factor
THA: 1,2,3,4-tetrahydro 9-amino acridine
TNF: Tumor necrosis factor
Try: Tryptophan
TTBS: Tween-20 Tris buffered saline
UB: ubiquitin
WT: wild type

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Abstract

Amyloidosis is a chronic degenerative disease that is characterized by the deposition of fibrous ß-pleated amyloid proteins in various soft tissues and organs (Glenner 1980, Kisilevsky 1983 & 1987). Sporadic form of Alzheimer's disease is one of the most common forms of systemic amyloidosis, with the current prevalence of approximately 4 million cases in the United States and 6 to 8 million cases worldwide (Kisilevsky 1987, Coleman 1990).

Amyloidosis can be experimentally induced in animals. In the inflammation-associated mouse model of amyloidosis, the preamyloid phase, which usually lasts for a number of weeks, is shortened to 24 to 48 hr in the recipient mice upon the administration of amyloid enhancing factor (AEF) AEF activity is present in a much higher level in amyloidotic animals, as well as the amyloidotic tissues in humans (Werdlin and Ranlov 1966, Kisilevsky 1983, Ali-Khan et al. 1988, Alizadeh-Khiavi et al. 1988).

Using brain extracts from Alzheimer patients and liver and spleen extracts from our well-characterized alveolar hydatid cyst (AHC)-mouse model of amyloidosis (Ali-Khan et al. 1983b, Alkarmi and Ali-Khan 1984, Abankwa and Ali-Khan 1988a & 1988b, Alizadeh-Khiavi and Ali-Khan 1988, Du and Ali-Khan 1990) I have been able to purify ubiquitin (UB) and demonstrate its AEF activity in mice (Alizadeh-Khiavi and Ali-Khan 1990, Alizadeh-Khiavi et al. 1990a & 1990b). UB, a stress protein universally found in all eukaryotes, can function as a sentinel-like molecule in the degradation of short-lived or abnormal proteins (Rechsteiner 1987). At a level as low as 10 μg, the tissue derived UB shows potent AEF activity in the mouse bioassay. The fact that recombinant wild type ubiquitin (R-UB) possesses AEF activity, that the AEF activity of both crude and R-UB is abolished by anti-ubiquitin antibody, and that the same antibody inhibits the in vitro amyloidogenic activity by ubiquitin-rich macrophages, further establishes the role of ubiquitin in amyloidogenesis. Using immunohistochemistry and immuno-electron microscopy I have confirmed the cell origin of AEF/ubiquitin, localized its presence in the intracellular compartments of polymorphonuclear leukocytes and macrophages, and demonstrated its binding to murine AA amyloid.

Resume

L'amylose est une condition chronique caracterisee par la deposition de proteines fibreuses amyloides B-pleated dans les divers tissus et organes (Glenner 1980, kisilevsky 1983 &1987). La maladie sporadique d'Alzheimer est le type le plus commun d'amylose systemique. Il y en a presentement 4 millions personnes qui ont la maladie d'Alzheimer, et 6 a 8 millions a travers le monde (Kisilevsky 1987, Coleman 1990).

Un modele experimental de l'hydatose alveolaire de souris m'a permis de caracteriser un agent d'augmentation de l'amylose (AEF). La phase pre-amylose de ce modele dure normalment un nombre de semaines, mais l'induction d'amylose est reduit a 24 a 48 hrs avec l'administration d'AEF. L'activite AEF est plus evident chez les animaux amyloidotiques, et dans les tissus amyloidotiques des humains (Werdlin and Ranlov 1966, Kisilevsky 1983, Ali-Khan et al. 1988, Alizadeh-Khiavi et al. 1988).

Les extraits des cerveaux des personnes ayant la condition Alzheimer, et les extraits du foie et de la rate provenant du modele de l'hydatidose alveolaire de souris (Ali-Khan et al. 1983b, Alkarmi and Ali-Khan1884, Du and Ali-Khan 1990) m'ont permis de purifier ubiquitin (UB) et de demontrer son activite d'augmentation d' amylose (AEF) chez les souris (Alizadeh- khiavi and Ali-Khan 1990, Alizadeh-Khiavi et al. 1990a and 1990b). UB, une proteine de stress des eukaryotes, a pour fonction la degradation des proteines anormales (Rechsteiner 1987). L'administration d'une concentration de 10 ug d' UB provenant de tissus des bioessais de souris abouti a l'activite amyloidogenique (AEF). Le fait que recombinant wild-type ubiquitin (R-UB) possede l'activite AEF, que l'effet d'activite AEF de l'UB brut et de R-UB est elimine par l'anticorps anti-ubiquitin, et que cet anticorps refreni l'activite amyloidogenique in vitro des macrophages riche en ubiquitin, demontre l'activite AEF de UB.

L'immunohistochimie et le microscope immuno-electron ont ete utilises pour identifier l'origine cellulaire d'AEF/ubiquitin, pour le localiser dans les compartments intracellulaires des leukocytes et des macrophages polymorphonucleaires, et pour demontrer chez les souris son lien avec l'amyloid A (AA).

GENERAL INTRODUCTION

Amyloidosis describes a heterogeneous collection of systemic diseases characterized by the extracellular deposition of ß-pleated amyloid fibrils of great chemical diversity in various organs and tissues (Glenner 1980, Cohen and Connors 1987, Kisilevsky 1987). Structural studies on purified amyloid proteins indicate at least 10 different chemical forms of human amyloids (Cohen and Connors 1987, Kisilevsky 1987). Proteolytic cleavage and release of amyloidogenic fragments from larger amyloid precursor proteins are believed to occur prior to amyloidogenesis in most forms of amyloid (Ericsson et al. 1987, Hoffman et al. 1984, Glenner and Wong 1984a &1984b, Linke et al 1989, Westermark 1982). However, a protease(s) which is presumably involved in the proteolytic processing of precursor proteins of various amyloids has eluded detection.

Despite significant diversities in the primary structure of amyloid proteins, all amyloids share a number of distinct staining, spectral and ultrastructural characteristics (Glenner 1980, Cohen and Connors 1987, Kisilevsky 1987). Whether or not various amyloids which develop in response to apparently disparate clinical settings, ranging from rheumatoid arthritis to Alzheimer's disease, share a common pathogenetic link remains a matter of much speculation and intense interest (Glenner 1980, Cohen and Connors 1987, Kisilevsky 1987, Hoffman et al. 1984, Glenner and Wong 1984a, Varga et al. 1986, Ali-Khan et al 1988b). Hall et al., to our knowledge, were the first to propose stress as the cardinal amyloid inducing factor in mice (Hall et al. 1960). Intermittent electrical shock, continuous fighting or over crowding, all stress-related conditions, were implicated to affect the incidence of amyloidosis in mice and Pekin ducks (Hall et al. 1960, Page and Glenner 1972, Cowan and Johnson 1970). However, in the absence of a definitive pathophysiological entity to tie amyloidosis as a possible sequel of stress, these earlier findings have remained buried in amyloidosis literature.

Recent studies indicate that in the mouse model of inflammation-associated experimental amyloidosis, one of two major apoprotein isotypes of serum A protein (SAA; an acute phase

protein), SAA_2, is the precursor protein of amyloid (Hoffman et al. 1984, Meek et al. 1986). Sustained high SAA levels during the preamyloid phase in conjunction with amyloid enhancing factor (AEF), is believed to predispose mice to amyloid deposition during the second stage of amyloidogenesis (Gertz et al. 1985, Kisilevsky 1983). The concept of AEF as an amyloid transfer or amyloid accelerating factor was first introduced by Werdlin and Ranlov (1966). Studies on AEF demonstrate a causative role for AEF in amyloidogenesis (Varga et al. 1986, Ali-Khan et al. 1988b, Kisilevsky 1983, Axelrad et al. 1982, Kisilevsky et al. 1977, Abankawa and Ali-Khan 1988a & 1988b, Alizadeh-Khiavi and Ali-Khan 1988, Axelrad and Kisilevsky 1980, Shirahama et al. 1990). AEF, although present in trace amounts in normal tissue extracts, increases markedly in spleens and livers of mice undergoing amyloidosis, and amyloid is never seen in murine tissues in the absence of elevated AEF levels (Axelrad et al. 1982, Abankawa and Ali-Khan 1988a, Axelrad and Kisilevsky 1980). A remarkable feature of AEF is its capacity, upon passive transfer in conjunction with one (Axelrad et al. 1982) or four (Varga et al. 1986, Ali-Khan et al. 1988, Niewold et al. 1986 & 1987, Yokota et al. 1989b) inflammatory stimuli, to induce accelerated splenic AA deposition in the recipient mice in 48 to 120 hr. Thus AEF is considered as a crucial pathogenetic factor in amyloidogenesis, although the mechanism by which it promotes accelerated amyloidogenesis is unclear (Varga et al. 1986, Ali-Khan et al. 1988, Kisilevsky 1983, Abankawa and Ali-Khan 1988b, Alizadeh-Khiavi and Ali-Khan 1988, Shirahama et al. 1990).

Recently, extracts prepared from amyloidotic tissues infiltrated with four chemically distinct forms of amyloids, A4 (brains; Alzheimer's disease and Down's syndrome), AL (spleen; primary amyloidosis), AA (spleen; reactive amyloidosis) and AF (spleen; familial amyloidosis), were shown to possess AEF activity (Varga et al. 1986, Ali-Khan et al. 1988b). This has led to

the concept that AEF might act as a "common denominator"and possibly a pathogenetic link in chemically diverse forms of amyloidosis (Varga et al. 1986, Ali-Khan et al. 1988a & 1988b). Attempts to purify and chemically identify AEF until now have been unsuccessful (Alizadeh-Khiavi and Ali-Khan 1990, Alizadeh-Khiavi et al. 1990a & 1990b). Based on the homogeneous preparations of AEF from alveolar hydatid cyst (AHC)-induced and mice, its partial amino acid sequence analysis, as well as the results from passive transfer experiments using anti-bovine ubiquitin IgG antibody, we present evidence that AEF activity is associated with ubiquitin (UB) (Alizadeh-Khiavi et al. 1990b). Subsequent to the present discovery, we extended this work to Alzheimer brain derived AEF; brain ubiquitin was purified, partially sequenced, biologically characterized and was found to be ubiquitin (Alizadeh-Khiavi and Ali-Khan 1990, Alizadeh-Khiavi et al. 1990a). Presence of AEF activity in recombinant wild type ubiquitin, in vivo abolition of AEF activity in recombinant UB by anti-ubiquitin antibody, and in vitro antibody mediated inhibition of amyloidosis by cultured UB-rich macrophages and neutrophils further emphasizes the role of UB in amyloid fibril formation.

Ubiquitin is one of the heat-shock (stress) proteins, present universally in all eukaryotic cells, and is known to participate in the degradation of abnormal or altered cytosolic proteins in stressed cells (Rechsteiner 1987 & 1988, Monia et al. 1990; reviews). Recent studies also indicate binding of UB to murine AA amyloid (Chronopoulos et al. 1990a & 1990b, Alizadeh-Khiavi et al. 1990b) and to a number of fibrillar proteins in diverse neurological and non-neurological degenerative diseases (Grundke-Iqbal et al. 1989, Perry et al. 1989, Heggie et al. 1989, Lowe et al. 1990a & 1990b). Furthermore, by immunohistochemistry and immunoelectron microscopy, using anti-mouse AA amyloid antibody, the AD-ubiquitin induced amyloid was identified as AA-type (Alizadeh-Khiavi et al. 1990a, Chronopoulos et al. 1990a & 1990b).

1.1 Alveolar Hydatid Disease

Hippocrates described hydatid disease as "a condition in where the liver is filled with water and bursts and the belly fills with water and the patient dies" (Jones 1948-53). Hydatid disease and hydatidosis are terms which currently refer to the infection with the metacestode stage of *Echinococcus* species. Alveolar hydatid disease (AHD) is caused by the larval metacestode of *Echinococcus multilocularis* (Leukart 1863, Virchow 1855, Rausch and Schiller 1956, Vogel 1957). 16 species and 13 subspecies of *Echinococcus* have been described so far. However based on the morphological features, only the four species *E. granulosus*, *E. multilocularis*, *E. oligarthus*, and *E. vogeli* are currently recognized as taxonomically valid groups (Kumaratilak and Thompson 1982).

1.1.1 Morphology of *E. multilocularis*

1.1.1.1. Adult Worm

E. multilocularis is a flat worm in its definitive hosts and belongs to the class Cestoda. Its size ranges from 1.2 to 4.0 mm and consists of a scolex with four suckers and a rostellum of double crown hooks, a neck which generates proglotids, and a chain of immature to mature proglottids (Thompson 1986) (Fig. 1.1.A). It is a hermaphrodite worm. The mature proglottid has a genital pore anterior to its middle and contains 15 to 30 testes, as well as ovaries, Mehlis and vitelline glands (Schmidt and Roberts 1985). Gravid proglottid containing eggs can detach from the rest of the worm and are excreted in the stool of the definitive host.

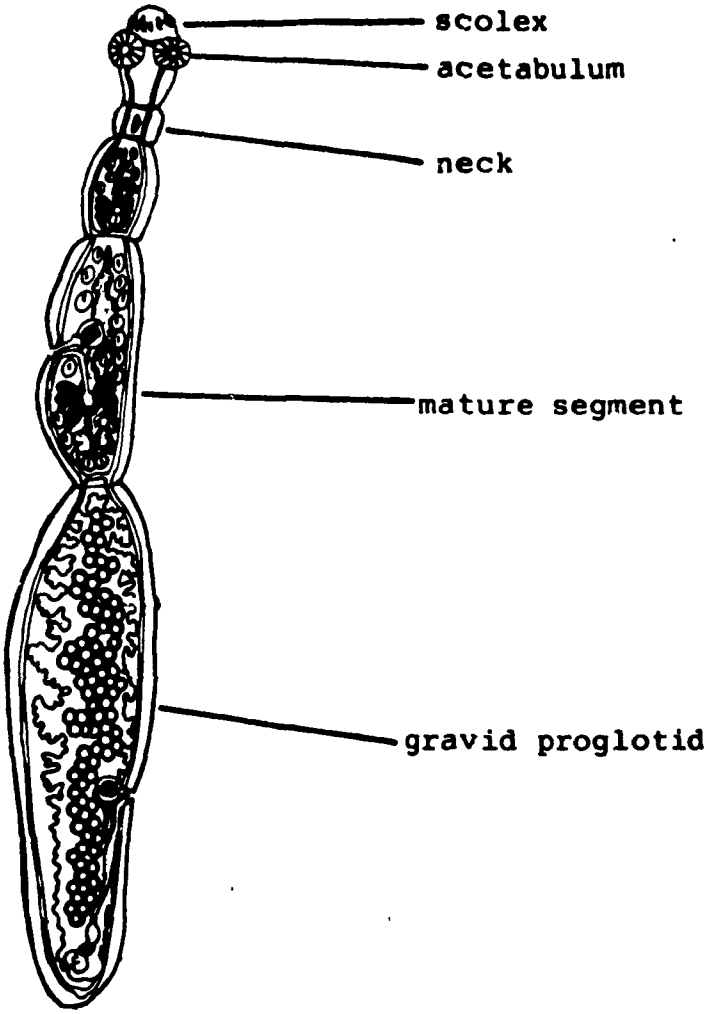


Fig. 1.1.A Mature *E. multilocularis*

1.1.1.2 Egg

Eggs are spherical to ovoid in shape and measure from 30 to 50 μm , by 22 to 44 μm (Lethbridge 1980). *E. multilocularis* eggs are indistinguishable from those of *E. granulosus* and various *taenia* species (Morseth 1965, Sakamoto 1981) (Fig.1.1.B). Embryophore is the main layer to protect the hexacanth oncosphere. Various layers of the shell also contribute to the extreme resistance of the ovum to a wide range of temperatures (Lethbridge 1980). Eggs can survive at -26°C for 2 months (Schiller 1955).

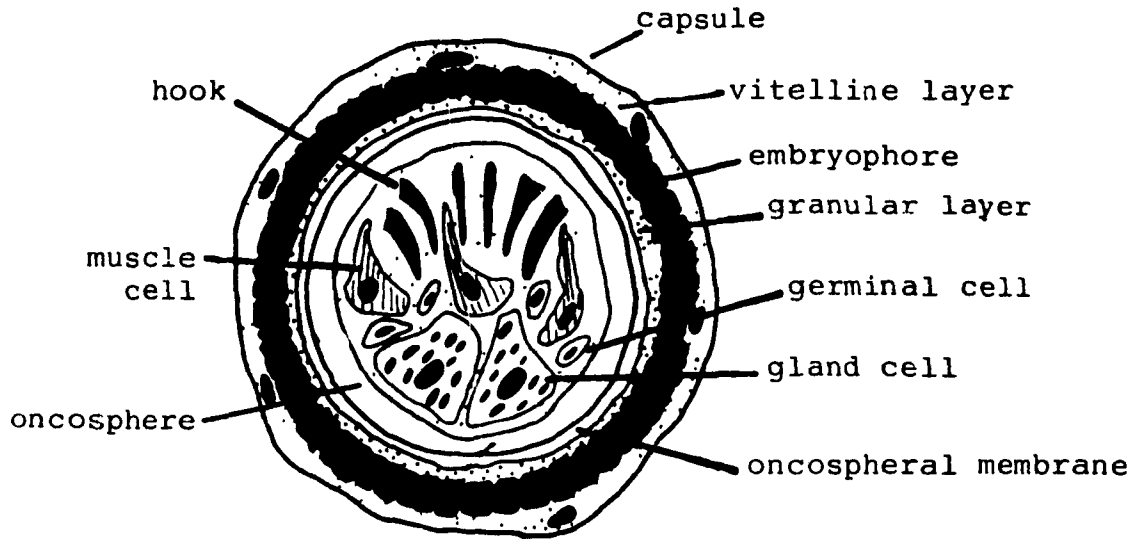


Fig. 1.1.B Ovum of *E. multilocularis*

1.1.1.3. Metacestode

Metacestode development in *E. multilocularis* is different from *E. granulosus* (Wilson and Rausch 1980). The larval stage which is also known as alveolar hydatid cyst (AHC) is a multicompartmental structure . It consists of numerous small vesicles embedded in a dense stroma of connective tissue (Fig. 1.1.C). The germinal layer is structurally similar to the tegument of the adult worm and consists of a distal cytoplasmic layer and a proliferative or germinal cell layer. Germinal layer cells can proliferate both endogenously and exogenously (Sakamoto and Sugimura 1970). Protrusions of the germinal layer from a network of filamentous

solid structures is responsible for infiltrating growth. These structures transform into new cysts later on (Vogel 1978, Ecker et al. 1983). Furthermore, germinal cells can detach from the germinal layer and metastatize via lymph or blood vessels to give rise to the distant metastatic foci characteristic of *E. multilocularis* (Ali-Khan et al. 1983a, Mehlhorn et al. 1983). Germinal cells also permit the maintenance of secondary hydatidosis in rodents by their repeated intraperitoneal passages (Alkarmi and Ali-Khan 1984, Ali-Khan 1974a & 1974b).

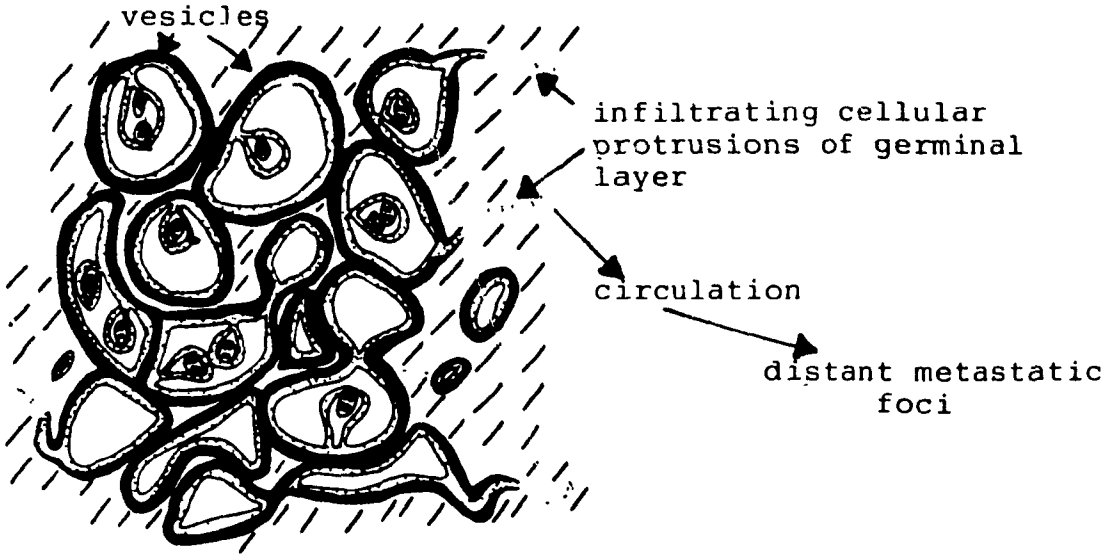


Fig.1.1.C Metacestode in connective tissue

1.1.2. Life Cycle and Epidemiology in Animals and Humans

Echinococcus multilocularis has been intensively studied in the field of epidemiology. Various species of rodents are infected by the larvae. The most important definitive hosts are foxes (genera *Vulpes* and *Alopex*) (Rausch 1986). In residential areas, dogs and cats (*Felis silvestris F. catus*) are the definitive hosts in the cycle. Dogs particularly are the main source of infection for humans. Wild cats and coyotes may also serve as final hosts (Leiby et al. 1970, Vogel 1961). In the intermediate host, the embryo often migrates to the liver and develops into cyst structures.

It then spreads to other soft organs and organs through metastasis. Foxes and dogs eat the cyst-contaminated flesh, and develop the larvae into the adult worms in their intestine (Fig. 1.2). *E. multilocularis* lives as the adult stage worm in the small intestine of the final host. Eggs are excreted from dogs or foxes and are the source of infection for numerous species of herbivorous and omnivorous intermediate hosts including humans.

Attempts have been made to delineate the geographic distribution of *E. multilocularis*. The natural cycle changes very little from place to place. Throughout the tundra, the natural cycle is based on the predator-prey relationship between arctic foxes, *Alopex logopus*, and rodents of the genera *Microtus*, *Lemmus*. On the St Lawrence Island, northern red-backed voles are highly infected as well (Rausch 1986).

The natural cycle in central Europe involves the red fox and rodents. Up to 40 percent of the foxes in southern Germany and northern Switzerland are infected (Vogel 1961). In central France, approximately 7 percent of foxes carry the cestode (Petavy and Delblock 1980). However, the infection is generally less frequent in rodents in the rest of Europe (Vogel 1961).

The life cycle of *E. multilocularis* in central North America involves the red fox and coyotes as final hosts; up to 70 percent of red foxes in North Dakota were found to be infected (Leiby and Olsen 1964, Leiby 1965).

E. multilocularis infection occurs rarely in the wolf (Rausch 1986). In Canada, both the larval stage and the adult parasite have been reported in rodents and foxes, from Manitoba, Saskatchewan, and Alberta (Hanatiuk 1966, Lubinsky and Galauger 1969). The spread of the parasite in the Japanese red fox is very well documented (Rausch 1967, Iida 1969, Kamiya and Ohbayashi 1975). *E. multilocularis* is expanding its range in Japan and in central North America, and its further geographic spread appears to depend on the inadvertent introduction of infected carnivores. An example of this in North America is capturing the red foxes in endemic areas and releasing them some hundreds of kilometres away for hunting purposes (Rausch 1986).

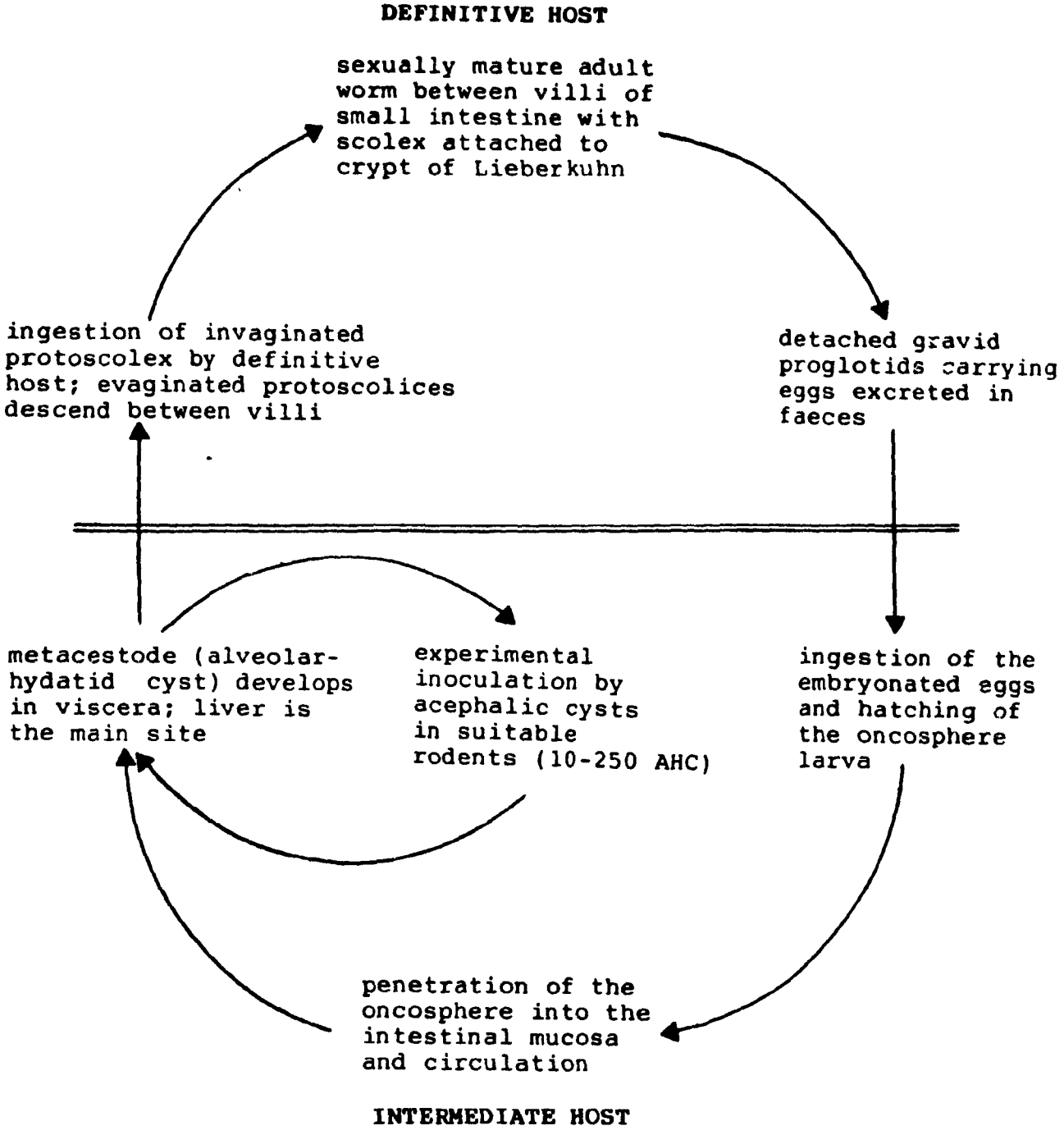


Fig.1.2. Life cycle of *E. multilocularis*

Many human cases of alveolar hydatid disease have been diagnosed in China (Jiang 1981, Han et al. 1981), and in India (Aikat et al. 1978). *E. multilocularis* has also been found in the Middle East countries: Turkey (Tahsinoglu and Hacihanefioglu 1962) and Iran (Alavi and Maghami 1964, Mobedi and Sadidhian 1971). Robbana et al (1981) reported a human case of AHC in northern Tunisia, the first indication of the potential spread of *E. multilocularis* in northern Africa.

It is important to note that humans carry the proliferating larval form of *E. multilocularis* without forming the protoscolices (Wilson and Rausch 1980).

1.1.3. Alveolar Hydatid Disease in Humans: Diagnosis and Treatment

Most cases of AHD are probably misdiagnosed as hepatic carcinoma. These tumor-like structures, 2 to 4 mm in diameter, (in radiology) have a radiolucent appearance outlined by calcium deposits in the hepatic tissue. CT scans or ultrasound also show similar lesions suggestive of AHD (Plorde 1983). Although cyst material of *E. multilocularis* have been reported to be poor antigens (Kagan et al. 1960, Kagan and Norman 1961), serological assays such as ELISA and indirect hemagglutination tests are used in the diagnosis of AHD in humans (Gottstein et al. 1983, Ali-Khan 1974a, Ali-Khan and Siboo 1982). Indirect hemagglutination test is positive in 90 percent of patients with hepatic cysts but in only 50 to 60 percent of those with pulmonary hydatidosis.

Specific diagnosis of AHD is based on histological examinations. Surgical treatment remains the standard therapy, and chemotherapy is not very effective against AHD (Wilson and Rausch 1982, Schwartz 1979). Mebendazole may be considered in patients with other medical problems that preclude surgery or in patients with extensive AHD. The drug is contraindicated in pregnancy (Plorde 1983).

1.1.4. Immunobiology and Immunopathology of Alveolar Hydatid Disease

There is very little known on serum antibody response to *Echinococcus multilocularis* adult worms (Kamiya et al. 1980, Kassis 1977, Kassis and Tannner 1976, 1977a). Moreover, whether worm's survival in the carnivore intestine is due to the induction of specific cellular unresponsiveness remains to be determined (Heath 1986).

On the other hand, the possible role of host immunological factors regulating the proliferation of *E. multilocularis* cysts in the intermediate host have not been studied sufficiently to explain the course of infection in permissive and restrictive hosts (Ali-Khan and Siboo 1980). The host cellular response to AHC is different in the invading oncosphere infection than in experimental secondary echinococcosis (Smyth and Heath 1970, Ali- Khan and Siboo 1980). With oncospheres, the necrosis of surrounding cells is followed by infiltration of neutrophils and macrophages. Occasionally eosinophils are also involved . The oncosphere may escape the cellular immunity during the development of the laminated layer . A thick layer of hyaline fibrous connective tissue eventually surrounds the older cysts. The larval cyst mass grows progressively and spreads despite the proliferation of B-cells in lymphoid tissues . Although parasite growth leads to the depression of the cytotoxic cellular response, humoral response remains functional. Histiocytes and giant cells replace the neutrophils around the cyst in the chronic infection.

In experimental secondary AHC infection, humoral response correlates with the size of the parasite biomass (Hinz 1973, Ali-Khan 1974b). Circulating low affinity IgG1, IgM antibodies, and to a lesser extent specific antibodies IgG2a and IgG2b, increase significantly during the progressive infection (Ali-Khan and Siboo 1982). Despite all this, the specific humoral response remains intact, since the infected mice show haemagglutinin and plaque forming cell response when challenged with sheep erythrocytes (Ali- Khan 1974a, 1979). IgG and IgM antibodies, as well as C3 have been detected on the surface of the cysts (Ali-Khan and Siboo 1981), and there are indications that antibody dependent cell cytotoxicity mechanism may play a role in regulating the growth of the parasite biomass during the initial stages of the infection (Ali-Khan et al. 1983b).

Kassis and Tannner (1977b) have shown that cystic stage of *E. multilocularis* infection is associated with depletion of the serum complement. This may be partly due to the anticomplementary effect of released calcareous corpuscles from protoscoleces of the parasite after lysis. Both classical and alternative pathways of complement activation are involved in this depletion (Kassis and Tanner 1976, Kamiya et al. 1980, Herd 1976). Kamiya et al. (1980) suggested that resistance to AHD in various hosts correlates with the level of the serum complement. Although complement components have some protective properties, they do not seem to abrogate the parasite proliferation.

Alveolar hydatid disease is a chronic granulomatous disease. In humans, the liver is the primary site of infection. The lesion is characterized by a central zone of necrosis surrounded by a dense fibrous tissue which is infiltrated with granulocytes and mononuclear cells (Rausch and Schiller 1956). The course of infection is accompanied by an elevation of neutrophils (Ali-Khan 1974b, Kroeze and Tanner 1986), and a decrease in the circulating eosinophils (Kroeze and Tanner 1986). Neutrophils and macrophages bind to the laminated layer, and by releasing their hydrolytic enzymes cause damage to the laminated and germinal layers of the parasite. Eosinophils are present, although to a lesser extent at the periphery of the cyst (Ali-Khan et al. 1983b). It appears that macrophages are the major effector cells in controlling the infection, since *Bacillus Calmette-Guerin* (BCG), phytohemagglutinin and *Taenia crassiceps*, which activate these cells, cause resistance to AHC infection in experimental animals (Rau and Tanner 1976, Baron and Tanner 1977, Reuben et al. 1978, 1979, Reuben and Tanner 1979, 1983).

Immune complexes (ICs) play a significant role in secondary pathology of *Echinococcus* infection (Ali-Khan and Siboo 1981, Ibarrola et al. 1981, Vialtel et al. 1981, Richard-Lenoble et al. 1978, Pini et al. 1983). ICs are detected in the infected mice sera as early as 4 weeks postinfection (p.i.). They get deposited in kidneys by 8 weeks p.i. and accumulate to form heavy deposits by 14 weeks p.i. (Ali-Khan and Siboo 1983). Immune complex deposits have also been

found in kidneys of humans infected with *Echinococcus* (Ozeretskovskaya et al. 1978, Ibarrola et al. 1981, Vialtel et al. 1981, Ali-Khan and Siboo 1983). ICs may activate the suppressor cells (Rao et al. 1980) and/or stimulate nonspecific degranulation of FC receptor-bearing cells (Taichman et al. 1972, Turner et al. 1973). When deposited in the kidney, ICs may initiate complement mediated damage to glomerular capillaries and subsequently cause glomerulonephritis (Aikat et al. 1978, Wilson and Rausch 1980). ICs may also be responsible for the disorganization of lymphoid tissues and T-cell depletion (Ali-Khan 1978, Ali-Khan and Siboo 1980, 1983).

1.1.5. Secondary Amyloidosis in Alveolar Hydatid Disease (AHD)

AA type of amyloidosis is usually associated with chronic granulomatous diseases such as tuberculosis, leprosy, and osteomyelitis (Cohen 1967, Glenner 1980, Mazur 1989). Amyloid deposits were first found in *Leishmania* infected hamsters and humans (Gellhorn et al. 1946, Hinglais and Montera 1964). It has also been reported in rodent filariasis (Growel and Votava 1975), human schistosomiasis (Andrade and Rocha 1979, Barsoum et al. 1979) and rodent and human alveolar hydatidosis (Ozeretskovskaya et al. 1978, Ali-Khan et al. 1983a & 1983b, McAdam et al 1980, Ali-Khan and Rausch 1987). High incidence of amyloidosis and death primarily due to kidney failure was recorded in patients with metastatic form of alveolar hydatidosis.

Experimentally induced AHD infection in mice provides an excellent model for the study of secondary amyloidosis, since it induces amyloidosis in less than a week and unlike other methods does not require multiple administration of the amyloidogen (Ali-Khan et al. 1983b, Alkarmi and Ali-Khan 1984). The marginal zone of white pulp, and the red pulp area in the spleen are the primary sites for amyloid deposition in AHC infected mice, although it occurs in the liver, kidney, and other soft organs later on (Alkarmi and Ali-Khan 1984). Balb/C and C57BL/6J mice are more susceptible to amyloidosis, while A/J mice are resistant to amyloidosis (Alkarmi and Ali-Khan 1984).

Purified AHC-induced amyloid shows similarities and some differences with the published data from mouse casein-induced AA amyloid. Small amounts of methylated basic amino acids and amino sugars are also present in AHC-induced amyloid (Alkarmi et al. 1986). AHC-induced amyloid is a 8.7kD protein with a pI value of 5.3 to 5.8 (Alkarmi et al. 1986).

Amyloid enhancing factor (AEF) activity, an essential factor for amyloidogenesis (Kisilevsky 1983), is found in abundance in murine amyloidotic livers and spleens (Abankwa and Ali-Khan 1988a and 1988b).

1.2. Alzheimer's Disease

With the current prevalence of approximately 4 million in North America and 6 to 8 million worldwide, Alzheimer's disease is the most common cause of dementia in adult life (Coleman 1990, Constantinidis 1978, Terry and Davies 1980, Katzman 1976, Tomlinson 1977, Wang 1977, Johnson et al. 1979). Worldwide approximately 10 percent of people over 65 develop Alzheimer's disease, while almost 40 percent of individuals over age 85 have AD. This latter age group is the fastest growing segment of population in North America (Mortimer et al.1981). Senile dementia of Alzheimer type (SDAT) is more common among women, perhaps because they live longer than men (Katzman 1986).

According to the data from Alzheimer's Society of Canada and Statistics Canada in 1986, 7 to 10 percent out of 2.7 million over the age of 65 suffer from Alzheimer type dementia. At least 20 percent of the population over 80 in Canada are afflicted with dementia, and approximately 50 percent of this population have Alzheimer's disease (Statistics Canada. Annual projections of the population by age and sex, Canada, Provinces, and Territories, 1984 to 2006. 1984; Division de la sante mentale, Sante et Bien-Etre Social, Canada. La malade d'Alzheimer: renseignemets a l'intention des familles, 1985). Citizens over 65 will constitute 14 percent of the Quebec population in year 2000 and will increase to the estimated value of 27 percent in the

year 2031. However, the widely quoted statistics of 100,000 to 300,000 living Canadians currently suffering from the disease appears somewhat exaggerated in these studies (Gautrin et al. 1990). More accurate estimations of 73,696 cases of AD occurring in 1986, and a projective estimate of 215,847 cases in 2031 have been recently reported (Gautrin et al. 1990).

The recognition of the disease as a clinical syndrome and the related pathological findings were first established by Alois Alzheimer (1907a, 1907b). Initially AD was believed to be a presenile form of dementia. Subsequently the association between the density of senile plaques (consisting of amyloid and dystrophic neurites) and the severity of the dementia was demonstrated irrespective of age (Blessed et al. 1968, Tomlinson et al. 1970). There appears to be two forms of Alzheimer's disease, an early onset type that strikes people in their fifties, and a late developing type which appears in the seventh or eighth decade of life. Some presenile or early onset AD cases are shown to be hereditary and caused by an abnormal gene which is located in a nearby locus to ß-amyloid gene on chromosome 21 in humans (Jean Mark 1990). Cloning and sequencing of ABPP gene from patients with Hereditary Cerebral Hemorrhage Dutch type (HCHWA-D) have revealed a cytosine to guanine mutation that causes a single amino acid substitution (glutamine instead of glutamic acid) at position 22 of the amyloid protein in the vessel walls of the brain of these patients (Van Broeckhoven et al. 1990, Levy et al. 1990a & 1990b, Prelli et al. 1988, Van Duinen et al. 1987). The same amyloid protein was isolated from brains of aged individuals with Down's syndrome (Glenner and Wong 1984a & 1984b, Masters et al. 1985a & 1985b, Beyreuther et al. 1986), and also from the brains of individuals with Guamanian Parkinsonism-dementia (Guiroy et al. 1987).

1.2.1. Neurofibrillary Tangles (Intracellular Amyloid) in Alzheimer's Disease

Atrophy in the temporal cortex of the brain is a common pathological finding in Alzheimer's disease (Hubbard and Anderson 1981), and is believed to reflect the loss of cortical neurons. Number of large cells within the cerebral cortex are reduced and the large pyramidal neurons show a predilection for neurofibrillary tangle (NFT) formation (Terry et al. 1981). Such pathological changes are also seen in the brains of Down's syndrome patients who live beyond the age of 40. NFTs are large masses of abnormal fibres which are commonly present in the perikarya of neurons in the hippocampus (Ball 1977), the upper three layers of the frontal and temporal neocortex (Simchowitz 1911, Goodman 1953, Corsellis 1977, Tomlinson 1977), the substantia innominata (Hirano and Zimmerman 1962), the amygdala (Jamada and Mehraein 1968), and in several brain stem nuclei (Jamada and Mehraein 1986).

Ultrastructurally, tangles in the perikarya of nerve cells are made of paired helical filaments (PHF) (Kidd 1963, Terry 1963, Wisniewski et al 1976) with the half-periodicity of 80 nm and widths ranging from 10 nm to 25 nm. PHF has been the subject of chemical investigation for over 15 years, yet its nature remains unclear. Due to the lack of purity, X-ray diffraction studies have failed to define the nature of the PHF (Selkoe 1981). Based on immunohistochemical and/or chemical analysis, the following proteins are found to be present in the PHF structure: neurofilament protein (Dahl et al. 1982), MAP2, τ (Wood et al. 1986), ubiquitin (Mori et al. 1987), and amyloid ß-protein (Glenner 1984a). PHFs are partially composed of cytoskeletal protein τ (Wischik et al.1988a, 1988b, Kowall and Kosik 1987); however up to 90 percent of the mass of the filament core has yet to be identified. Two τ peptides (9.5kD and 12kD) have been purified from the pronase digested filaments and sequenced (Goedert et al. 1988). cDNA corresponding to the 352 residue parent molecule of these two peptides has been isolated, showing homology to the amino acid sequence of τ purified from mice microtubles (Lee et al. 1988). PHF is highly insoluble even in stringent denaturing conditions. This subsequently makes

it very difficult for any chemical analysis. It is not clear yet whether NFTs are, in themselves neurotoxic or whether their excessive presence within the nerve cells and its processes are likely to impair the neuronal function. The degree of accumulation of PHF in NFTs and cell bodies correlates very well with the severity of the AD features , including the degree of dementia (Tomlinson et al. 1970, Tomlinson and Henderson 1976, Wilcock and Essiri 1982), extent of neuronal loss in the hippocampus (Ball 1977), and the degree of cortical choline acetyltransferase deficiency (Perry et al. 1978, Wilcock et al. 1982). NFTs are also found in a wide variety of neurological disorders other than AD (Iqbal and Wisniewski 1983, Joachim et 1987) such as Guam Parkinson Dementia , post encephalitic Parkinson's disease, long-surviving cases of subacute panencephalitis, and Haller-Vorden-Spatz disease. The presence of diffuse altered neurites and PHFs similar to that present in AD, although without any amyloid deposits, has been demonstrated in subacute sclerosing panencephalitis (SSPE) (Tabaton et al. 1989). These findings clearly suggest that widespread alteration of neurites in AD may be unrelated to amyloid deposition. Significantly, some of the dystrophic cortical neurites that make up senile plaques also contain PHF (Tabaton et al. 1989).

Straight filaments varying in diameter from 10-20 nm are also seen in some NFTs (Metuzals et al. 1981, Selkoe 1984, Shibayama and Kitoh 1977, Yagshita et al. 1981, Yoshimura 1984). These straight filaments can coexist with PHFs in neurites. Straight and helical filaments may be chemically related.

1.2.2. Neuritic Plaques

Neuritic plaques (NPs) are abnormal structures measuring up to 200 μm in size, and are seen in the cortex, hippocampus, subiculum, hippocampal gyrus, and amygdala (Jamada and Mehraein 1968, Tomlinson 1977). They are mainly made of dystrophic neurites and amyloid. NPs stain as dark bodies with knob-like structures by the silver impregnation technique. Immature plaques may not contain amyloid, while amyloid gets deposited later on (Price 1984). NPs show

increases in oxidative enzymes (Friede 1965) and acid phosphatase activity (Josephy 1949). α-1 antichymotrypsin is also a component of NP and cerebrovascular amyloid (Abraham et al. 1988). Astrocytes and microglial cells are often present in the vicinity of plaques (Wisniewski et al. 1989). Ultrastructural examination of plaques reveals that neurites are made up of unmyelinated axons and nerve ends containing PHF, dense bodies, and mitochondria (Kidd 1964, Terry et al. 1964, Gontas et al. 1967). Amyloid in plaques appears as extracellular 9-10 nm filaments. Amyloid deposits are also present around blood vessels in AD brains, a condition known as congophilic angiopathy (Mandybur 1975).

As in the case of NFTs, there is also strong correlation between the numbers of neuritic plaques and the severity of dementia in AD. NPs and NFTs are major histological features in AD (Corsellis 1977, Tomlinson 1977). Some monkeys also develop neuritic plaques with increasing age (Wisniewski and Terry 1973, Wisniewski et al. 1973). Cerebrovascular amyloid is always the most abundant form in squirrel monkeys, while aged rhesus monkeys (*Macaca mulatta*) develop mostly parenchymal deposits of amyloid along with relatively less vascular amyloid (Walker, L. C. et al. 1990). Whether this is related to plaque formation in AD patients remains unclear (Wisniewski and Terry 1973).

1.2.3. Extracellular Amyloid in Alzheimer's Disease

Although AD was first thought to be a disease characterized by a brain-limited amyloidosis (Ogomori et al. 1989, Scudiero et al. 1986, Peterson and Goldman 1986, Zubenko et al. 1987), Joachim and others (1989) have lately reported the detection of amyloid ß-protein (Glenner and Wong 1984a) or A4 (Masters et al. 1985a) deposits in non-neuronal tissues of AD patients including skin, subcutaneous tissue and intestine. Thus, amyloidosis in AD is systemic in nature.

ß amyloid or A4 protein has a relative molecular mass of 4.2kD and consists of 39 to 43 amino acids (Glenner and Wong 1984b, Selkoe 1986, Masters et al. 1985a, Selkoe et al. 1988). It is a hydrophobic subunit of a much larger precursor protein (Fig. 1.3) (Kang et al. 1987, Pont et al 1988, Selkoe et al 1988, Tanzi et al. 1987 & 1988, Kitaguchi et al. 1988, Weideman et al. 1989), and forms very stable 5-10 nm filaments (Gorevic et al. 1987).

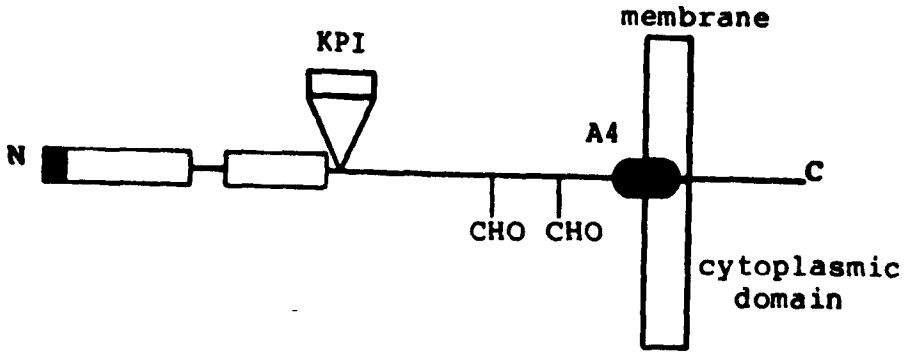


Fig.1.3. Schematic diagram of ABPP molecule

The factors responsible for the generation of A4 amyloid from ABPP in AD have not yet been determined. ABPP is known to contain a primary structure motive known as PEST sequence (Proline-Glutamic acid-Aspartic acid-threonine) which is a distinguishing feature of most protease sensitive rapidly turning over proteins (Rechsteiner et al. 1987, Simon and Christoph 1989). Normal processing of ABPP does not produce amyloid deposits, while altered ABPP processing have been suggested as the key pathogenic event. This hypothesis has been supported by Esch et al. (1990) who showed that constitutive processing of ABPP involves a cleavage in the interior of the A4 region, thus preventing amyloidogenesis (Fig. 1.3). Therefore

a deficient or abnormal processing may be the etiological event in AD. Interestingly amyloid-like fibrils have recently been reported in COS cells overexpressing part of the Alzheimer's amyloid precursor protein (Maruyama et al.1990).

ABPPs (Fig.1.3) are 108 to 136 membrane associated N- and O- glycoproteins (Selkoe et al. 1988, Weideman et al. 1989), with a large extramembranous portion and a small cytoplasmic domain (Kang et al. 1987). Mature forms of ABPP undergo tyrosine sulphation and are inserted at the cell surface. The extracellular domain of the precursor protein is glycosylated at position 476-469 and 496-498 (Kang et al. 1987). The A4 sequence corresponds to residues 597-638/639 close to the C-terminal of ABPP 695 (Kang et al. 1987, Robakis et al. 1987), with part of it located in the transmembrane domain (residues 625-648).

There are three major forms of ABPP: ABPP695, ABPP751, and ABPP770 (Ponte et al. 1988, Tanzi eta l. 1988, Kitaguchi et al. 1988). The mRNAs for these are derived by alternative splicing of transcripts from a single gene (Kang et al. 1987, Goldgaberg et al. 1987, Tanzi et al. 1987, Robakis et al. 1987). Transcripts encoding 751 or 770 residue proteins (ABPP 751, 770) contain a 56 amino acid domain with a 50 percent homology to the Kunitz family of serine protease inhibitors (KPI). These transcripts and their products are expressed in neuronal and non-neuronal tissues as well as in different cell lines (Bahmanyar 1988, Autilio-Gambetti et al. 1988). Widespread expression of ABPP gene in rat and mouse brain has also been very well documented (Mita et al. 1989). The function of ABPP has not yet been determined. The protease inhibitory forms ABPP751, 770 (with KPI) are widely distributed in all tissues, while ABPP 695 is present only in the nervous system. Recent findings suggest that portions of ABPP may act as trophic factors in various cell types, or the secreted forms of ABPP may act as growth regulating molecules (Saitoh et al. 1989): fibroblasts transfected with an antisense ABPP cDNA produce less ABPP and grow poorly compared with control fibroblasts. Furthermore, the secreted N-terminal portion of ABPP with or without the KPI insert can act as growth factors for non-neuronal cells probably affecting cell adhesion (Saitoh et al. 1989). ABPP 751 but not

ABPP 695 is mitogenic for 3T3 fibroblasts in vitro. Allsop et al (1988) suggested that the amino-terminus portion of the ABPP could also act as an extracellular ligand and can promote cell survival in vitro (Whitson et al. 1989).

The amino acid sequence of ABPP shows identical homology with protease nexin-II, a protease inhibitor which forms stable complexes with the epidermal growth factor (EGF)-binding protein, the gamma subunit of nerve growth factor and trypsin (Van Nostrand et al. 1989). Potent anti-chymotrypsin effect of ABPP and association of α-1 antichymotrypsin (Abraham et al. 1988) with NPs suggests a possible altered proteolytic role in pathogenesis of AD. ABPP has also been shown to be identical to the platelet coagulation factor XIa-inhibitor (Smith et al. 1990)

1.2.4. Pathogenesis of Alzheimer's Disease

Senile dementia of Alzheimer type is not considered to be exclusively a genetic disorder. Recent statistical data suggests that AD is not a single entity, but rather results from genetic defects on chromosome 21 or from other nongenetic factors (St George-Hyslop et al. 1990). With the exception of Hereditary Cerebral Hemorrhagic amyloidosis of Dutch type (HCHWA-D) (Levy et al. 1990) there is no other ß fibrilosis with intergenic mutation in ABPP exons.

AD patients show no clinical abnormalities suggestive of aberrant metabolism of ABPP prior to the disease. Many neurons function normally throughout the life of the AD patients. This suggests that the pathogenesis of cerebral dysfunction in AD may be directly related to the deposition of amyloid fibrils in the vulnerable regions of the brain, rather than to fundamental disease-specific changes in the normal function or metabolism of ABPP.

Overproduction of ABPP may contribute to the A4 deposition in the brain of AD and Down syndrome patients, since there are differences in the total level of ABPP expression between AD and normal brains (Higgins et al. 1988, Schmechel et al. 1988, Palmer et al. 1988, Johnson et al. 1988, Tanaka et al. 1988, Klark et al. 1989, Anderson et al. 1989). Since amyloidosis is the earliest marker of Alzheimer's disease, it has been hypothesized that neuronal dysfunction in AD

is directly due to the deposition of amyloid between neurons (Glenner 1984b). Interestingly, others have shown that ß amyloid is essential for the survival of cultured cells. Yanker and others (1990) suggested that amyloid A4 protein may function as a neurotrophic factor for immature and differentiating neurons, but at a higher concentration it may cause degeneration of the mature cells. Furthermore, the ABPP mRNA level between trisomy 21 Down-syndrome patients and normal human controls with two chromosomes appears to be higher than the expected 3:2 ratio (Tanzi et al. 1987). Goldgaber et al. (1989) have suggested a role for interleukin-1 and several other mediators in the oversynthesis of ABPP. Gliosis is a prominent feature in AD and Down syndrome, and can be detected even before the neuronal changes occur (Meyer et al. 1939, Ellis et al. 1974, Wisniewski et al. 1985, Griffin et al. 1989, Mattiace et al. 1990). Glial cells and astrocytes type- I produce fibrillar form of ABPP in culture (Robakis et al. 1990). HLA-DR positive reactive microglia are embedded in the core of senile plaques (Itagaki et al. 1989, Mattiace et al. 1990). Increased IL-1 levels appear to be responsible for gliosis and thus to increase the synthesis of ABPP in AD and DS brains (Griffin et al. 1989, Goldgaber et al. 1989). Molecular genetic studies have also revealed that the promoter of ABPP gene contains sequences homologous to the consensus binding site of heat shock control elements (Salbaum et al. 1989). This may well explain overexpression of ABPP in brains of elderly people, since aging is a natural stress phenomenon (Hall et al. 1960).

Deficient or abnormal proteolysis processing of ABPP during the secretion of its large soluble NH2-terminal fragment may be a crucial factor in A4 amyloidogenesis in AD brains (Esch et al. 1990). Normal processing of ABPP includes its cleavage in a site within the A4 region and this mechanism may be missing in AD (Sisodia et al. 1990). This hypothetical aberrant processing of ABPP into amyloid fibrils emphasizes its inappropriate cleavage at the NH2 terminus. Therefore, accumulation of some membrane-bound form of ABPP that can not be efficiently cleared from the cell may be the origin of amyloid plaque formation.

Since heparin sulfate accumulates in neurons and in the ß amyloid protein containing lesions of AD and DS, Snow et al. (1990) suggested that this may indicate the possible interaction

between these two components which may result in the pathogenesis seen in these diseases. Dow et al.(1990) suggested that undersulphation of proteoglycans may be responsible for the neurite outgrowth in AD. Meanwhile it is shown that proteoglycans are associated with neurite growth activity, and their accumulation in AD may reflect a regenerative response in neurons (Riopelle et al. 1990)

Transforming Growth Factors , which are involved in cell regulation and cell differentiation, bind ABPP molecules containing KPI domain (Bodmer et al. 1990). Moreover, basic fibroblast growth factor, a potent neurotrophic heparin binding growth factor which may play a role in neuronal degeneration (Anderson et al. 1988), has also been suggested as a primary stimulus for the gliosis seen in AD (Stopa et al. 1990).

A defect in the phosphorylation / dephosphorylation system which leads to the accumulation of abnormal phosphate of τ in PHF has been proposed in Alzheimer neurofibrillary pathology (Bancher et al. 1991). Matson et al. (1990) hypothesized that the same cellular signalling mechanisms mediating the calcium regulated neuronal circuitry are closely involved in the abnormal neuronal function in age-associated neurodegenerative disorders. Some cytoskeletal calcium-induced changes in cell culture resemble those seen in AD, which may indicate the involvement of abnormal cellular signalling in the pathogenesis of this disease (Matson et al. 1990). Bunke et al. (1990) using synthetic peptides and tyrosine-kinase pp60v-src, have demonstrated an evolutionary conserved functional intracytoplasmic acceptor site, tyrosine 682 for phosphorylation in ABPP which may be involved in signal transduction and receptor recycling

1.2.4.1 Possible Environmental and Other Factors in Pathogenesis of Alzheimer's Disease

1) Viruses: An infectious hypothesis has been suggested for AD, however there is no evidence for transmission between humans, and attempts to transmit the disease to primates have failed with the exception of two cases (Roos 1981). Nonetheless, Scrapie associated fibrils have not been found in AD (Merz et al. 1984). Thus it seems improbable that a slow viral agent is

involved in the etiology of this disease. Interestingly, two distinct types of plaque, composed of different amyloids co-exist in the human slow viral disease; one related to PrP (Prion protein) and the other similar to Alzheimer plaque (Allsop 1986).

The role of common viruses in the etiology of AD associated with a particular genetic predisposition has also been suggested. For example herpes simplex virus (HSV) can cause encephalitis and subsequent formation of NFTs (Esiri 1988, Gautrin and Gauthier 1989).

2. Neurotoxic agents: Craper et al. (1973 and 1976) first reported high concentrations of aluminum present in NFTs and NPs of AD brains and other neurodegenerative disorders such as Down's syndrome, Parkinsonism, and amyotrophic lateral sclerosis. Aluminum accumulates in chromatin of the nucleus of affected neurons, in proteinous part of NFT, and in amyloid core (Krishnann et al. 1988). However its possible pathogenetic role in neurodegenerative disorders remains unclear (Gautrin and Gauthier 1989). Interestingly, in cell cultures, aluminum can potentiate the induction of all major heat shock protein species including ubiquitin, HSP 70, and HSP 28, which may have pathogenetic implications in AD (Omar and Pappola 1990). There is no evidence that aluminum ingested through antacid medication could cause pathological features seen in AD brains (Gauthrin and Gauthier 1989, Amaducci et al. 1986). On the other hand , neurological disorders have been reported in chronic hemodialysis patients (Alfrey et al. 1976), and the severity of the encephalopathy appears to be related to aluminum levels in brains of patients (Alfrey et al. 1976). Aluminum accumulates in lysosomes of glial cells in the brain of dialysis encephalopathy patients, however with no NFT or NP formations (Buge 1978). Rats injected systemically with aluminum chloride also show high levels of aluminum in their brains, but lack neuronal tangles and plaques (Galle et al. 1980).

Several other neurotoxic metals and trace elements including lead, tin, manganese, mercury and zinc have also been the focus of attention for the last few years, although their potential involvement in pathogenesis of AD remains unknown (Gautrin and Gauthier 1989). Since PHFs in AD brain are primarily detected in cortical grey matter neurons adjacent to the basement

membranes of capillaries, it is hypothesized that neurotoxins carried via blood vessels might lead to the degeneration of surrounding neurons in their close vicinity, causing the formation of PHF in affected cells.

3) Immune system dysfunction in Alzheimer's Disease: Involvement of autoimmunity in the pathogenesis of AD has been suspected for many years. Presence of IgG in senile plaques has already been reported by several groups (Ishii et al. 1975, Ishii and Haga 1976, Ihara et al. 1981). Parallel studies have demonstrated reduced ß-globin fraction, but normal IgG levels, in cerebrospinal fluid of AD patients compared to normal age-matched humans (Soininen and Heinonen 1982). Furthermore, autoantibodies to AD brain tissue have been demonstrated in the sera of these patients (Gaskin et al. 1987, Singh and Fudenberg 1986). Complement fragments C1q, C3b, C3c, C3d, and C4 have also been demonstrated in plaques of AD brains (Ishi and Haga 1984, Ishi et al. 1988, Eikelenboom and Stam 1982, Eikelenboom et al. 1980), while complement function is unchanged in patients sera. Because of the questionable nature of IgG present in plaques, it is suggested that unknown nonimmunological mechanisms may trigger the classical pathway of complement fixation (Eikelenboom et al. 1980). Membrane attack complex, C5-C9 is absent from senile plaques probably because of the lack of bilipid membrane, but coexists with dystrophic neurites and tangles (McGeer et al. 1989). Nevertheless, the significance of these findings has remained uncertain.

All the cellular elements required for the immune response are present in AD brain tissue. HLA-DR positive reactive microglia strongly associated with senile plaques and small number of infiltrated T-cells are present in AD brain (McGeer et al. 1989). A classical function of HLA-DR is to present foreign antigens to T-helper cells. However, HLA-DR antigens are also expressed in non-disease conditions as well, and are not necessarily indicative of cellular immune

activity. The importance of the reactive gliosis, and of the presence of T4 and T8 lymphocytes (Itagaki et al. 1988, Lube-Narod and Rogers 1988) as autoimmune pathology of AD is not yet clear. Infiltration of T-cells into the brain tissue in AD, complement fragments fixed on dystrophic neurites, and A4 amyloid also suggest that the blood-brain barrier is not essentially intact and might be damaged in this disease (Alafuzoff et al. 1987).

4) Nucleating factor: An autocatalytic nucleating mechanism, also known as amyloid enhancing factor (AEF) for amyloidogenesis in AD and other amyloidotic diseases has been proposed (Gajdusek 1988, Ali-Khan et al. 1988b). According to this hypothesis, AEF with its tendency to complex with itself or other molecules could serve as the nucleus for accelerated amyloid formation (Gajdusek 1988). Fibril associated AEF (Niewold et al. 1986 & 1987) is believed to be identical to ubiquitin (Alizadeh-Khaivi et al. 1990a & 1990b); ubiquitin associates with amyloid fibrils in mice (Chronopoulos et al. 1990a & 1990b, Alizadeh-Khiavi et al. 1990a) and may be involved in proteolytic cleavage of the precursor protein of AA type amyloid. AEF and its role in amyloidosis will be discussed in more detail later in section 1.4.7.

1.2.5. Diagnosis and Treatment of Alzheimer's Disease

There is no consistent genetic or biochemical marker for the diagnosis of AD. Diagnosis based on the clinical judgement is useful only when the patient is freely communicative (Richardson and Adams 1989). Clinical diagnosis of AD appears to be inaccurate, and approximately 20 to 30 percent of the cases are usually misdiagnosed. Although hematological tests in AD appear to be normal, major CSF biochemical changes are evident in that somatostatin, delta-sleep-inducing peptide and acetylcholine esterase are significantly decreased (Adolfson et al. 1990). Increased chromogranin A/ synaptophysin ratio in AD and Pick's disease has also been reported (Lassman et al. 1990). An exact diagnosis is based on postmortem examination of brain

samples for the identification and counting of neurofibrillary tangles, neuritic plaques and congophilic angiopathy. ß-amyloid also appears in non-neuronal tissues in a much higher frequency compared to normal aged individuals, and seems to be a promising diagnostic method in the future (Jochim et al. 1989).

An immunological assay for A68, a selective protein marker for AD (Van Hoesen et al. 1987, Wolozin et al. 1987, Wolozin and Davies 1987) has been devised (Ghanbari et al. 1990, Flament and Delacourte 1990). A68 , a presumed abnormally phosphorylated τ protein (Ueda et al. 1990), is detected by a monoclonal antibody known as ALZ-50 (Davies 1988). Since ALZ-50 sandwich enzyme immunoassay can recognize early pathologic changes specific for AD (Hyman et al. 1987) in more than 85 percent of AD cases and requires minimal brain tissue biopsy samples, it could be the best diagnostic choice for early or late AD in living individuals (Ghanbari et al. 1990). The possibility of α-1-antichymotrypsin as a possible biochemical marker in AD has been suggested lately (Matsubara et al. 1990).

Because of the extensive neuronal damage, AD is considered an incurable condition. 1,2,3,4-tetrahydro-9-amino-acridine (THA) have been shown to restore the memory function in AD patients (Summer et al. 1986), however the side effects are serious and include hepatotoxicity and renal failure. It is suggested that THA acts in neuronal membrane skeletons to strengthen protein-protein interactions (Butterfield 1990). CSF somatostatin, delta-sleep-inducing agent and acetylcholine esterase is significantly lower in AD compared to the age-matched control. Attempts to inject somatostatin into the brains of aged monkeys produced Parkinson-like syndrome (Adolfson 1990). It is impossible to restore the damaged areas of the brain in AD, however the possibility of preventing further deterioration by neurotrophic agents such as nerve growth factor remains to be explored (Mark 1990). The finding that NGF receptors are reduced in AD brains is supporting this hypothesis (Lee et al. 1990). It has been shown that NGF promotes the growth of the brain neurons that release the neurotransmitter acetylcholine (Mark 1986, Mobley et al. 1985, Taniuchi et al. 1986).

1.3. Stress Proteins

Mammalian stress proteins are divided into two major groups: heat shock proteins (HSP) which are elevated in response to higher nonphysiological temperatures or to heavy metals and have the approximate molecular masses of 8,23,32,56,72,73,90, and 110kD; and 75,80, and 100kD glucose regulated proteins (GRP) which increase in response to glucose or oxygen deprivation (Welch et al. 1989). The two groups of proteins are often regulated in an inverse manner. All these proteins bind to ATP in vitro (Welch and Feramisco 1985). Furthermore, there is considerable homology between some members of these two groups, e.g. between GRP100 and HSP90 (Surgan et al. 1986), suggesting that they may also be related in their function. Diverse functions of these proteins is based on their capacity to associate and modify other proteins. Members of HSP70 and HSP60 families are molecular chaperons which are involved in the unfolding and translocation of proteins (Deshaies et al. 1988, Chirico et al. 1988). One of the best examples of this is the binding of BiP, a member of HSP70 family to the heavy chain of immunoglobulin inside the cytoplasm (Haas and Wable 1983, Munro and Pelham 1986). While HSP70 also prepares certain proteins for lysosomal degradation (Chiang et al. 1989), ubiquitin actually participates in ATP-dependent cytosolic degradation of misfolded or abnormal proteins (Parag et al. 1987). HSP70 and HSP65 are present in *Mycobacterium leprae* and *M. tuberculosis* as well as the mammalian host cells. Whether HSPs are more beneficial to the host or to the pathogen remains unclear (Polla 1988). Elevated levels of HSP70 are also found in the synovial fluid of arthritis patients (Kubo et al. 1985). Autoantibodies to various HSPs and ubiquitin are present in autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and ankylosing spondilitis (Minota et al. 1988, Muller et al. 1988, Lakomek et al. 1984). It has also been demonstrated that various chronic stress conditions in laboratory animals can

cause amyloidosis (Hall et al. 1959 & 1960, Page and Glenner 1972). This may explain the high prevalence of amyloidosis in aged or fighting animals and aged humans, and thus stress has been considered as one of the etiological factors in amyloidosis (Hall et al. 1959, Page and Glenner 1972). Because of its likely role in accelerated amyloidogenesis (Alizadeh-Khiavi et al. 1990a & 1990b), ubiquitin will be discussed in further detail.

1.3.1. Ubiquitin

Ubiquitin (UB) was first purified from thymus as a B-lymphocyte differentiation factor (Goldstein et al. 1975), and since antibodies prepared against it reacted with diverse mammalian cells as well as yeast and plants, the protein was named "ubiquitous immunopoietic peptide". Schlesinger et al. (1975) detected adenylate cyclase stimulating activity in UB preparation and published its complete amino acid sequence . UB is a 76 residue peptide with X-ray structure resolved to 2.8 A. It has a compact globular conformation with its carboxyl terminal arg-gly-gly extended into the solvent (Vijay-Kumar et al. 1985). The molecule contains four strands of ß sheet plus a single α-helix with three and one half turns. NMR studies showed that UB remains folded at a pH range of 1 to 13 and at below 80°C (Cary et al. 1980). Extensive hydrogen bonding and a hydrophobic core may account for exceptionally high stability of UB in various conditions. Through its carboxy terminus, UB is joined to the ϵ-amino group of lysine 119 of histone H2A (Goldknopf and Busch 1977, Hunt and Dayhoff 1977). Ubiquitin is an unusual protein, first because it is a highly conserved molecule (there is merely a difference of three amino acids between UB from yeast and from man), and second because it is found both as free molecules as well as covalently linked to other proteins through the isopeptide bonds involving its carboxyl terminal glycine residue and the primary or ϵ-NH2 groups of the ubiquitinated proteins. While histones H2A and H2B appear to be ubiquitinated (West and Bonner 1980), histones H1, H3, and H4 are not ubiquitinated. Several other proteins, including lymphocyte homing factor (Siegelman et al. 1986, St John et al. 1986), platelet-derived growth factor, actin

and microfillament associated proteins are also conjugated to UB (Murti et al. 1988, Monia et al. 1990). Ubiquitin gene is present in certain members of togavirus family (Meyers et al. 1989), and Avian Leukosis Virus particles package free cytosolic ubiquitin from host cells (Putterman et al. 1990). However the importance of UB in viral pathogenesis remains unclear.

Binding of UB to proteins may have functional implications. However, many other intracellular proteins which are targeted for intracellular degradation are found to be ubiquitinated. The covalently linked ATP-dependent proteolysis factor (APF) in reticulocyte lysates (Ciechanover et al. 1980, Hershko et al. 1980) was demonstrated to be ubiquitin (Hershko et al. 1980).

1.3.1.1. Ubiquitin-Protein Ligase System

UB-substrate conjugation is considered to be one of the essential steps in the ATP-dependent proteolytic process (Pickart et al. 1988). At least three enzymes are involved in this activation and ligation step: 1) E1 activating enzyme with the molecular mass of approximately 100kD which binds to UB by a thiol ester bond in the presence of ATP. 2) E2, UB carrier proteins of diverse molecular weight which accept UB from E1 and form thiol ester bonds with UB. 3) E3, large (~200kD) ligase enzymes which accept UB from E2 and conjugate it to the target proteins destined for proteolysis (Pickart 1988). Recognition of the substrate for ubiquitination by E3 is based on the N-end rule which relates to the metabolic stability of the protein. The degradation signal comprises a destabilising basic or bulky hydrophobic amino-terminal residue and a specific internal lysine residue (Varshavsky et al. 1988). However in the case of proteins with acidic NH2 terminal amino acid, Post-translational addition of arginine moiety to this site is required for the recognition by E3s(Elias and Ciechanover 1990). Moreover, in the case of multimeric proteins, only the subunit bearing the lysine determinant is actually degraded (Johnson et al. 1990). Ubiquitinated target proteins can be attacked by a complex protease

machinery, such as macropain or megapain (Rechsteiner 1988). Following the ATP-dependent proteolysis, intact UB is released and is recycled (Hough et al. 1988). On the other hand, the isopeptide bond between UB and the target protein can be cleaved by isopeptidases or ubiquitin carboxyl-terminal hydrolases. There are many UB-hydrolases with specificity towards certain UB-substrate complexes. Most of hydrolases involved in this event remain to be discovered. Such a mechanism is presumed to involve the post-translational proteolysis of UBI4 gene product into monomeric UB (Rose 1988). It is suggested that UB may undergo conformational changes following conjugation to a target protein (Wilkinson and Mayer 1986). This is an attractive model, since it will reduce competition for components in the activating and proteolysis pathways. This model is supported by the evidence that some antibodies preferentially bind free or conjugated forms of the protein (Haas and Bright 1985).

There is also evidence that UB has intrinsic proteolytic activity comparable to that of other well-characterized serine proteases (Fried et al. 1987). If so then conjugation of UB to a protein may convert that protein into a specific protease and can play a role in certain cellular regulatory mechanisms. However, no enzymatic cleft is seen in the X-ray structure of UB. Moreover, the sequence of calpastatin, a calcium activated protease inhibitor shows partial homology with that of UB (Rechsteiner 1988). This suggests that UB may interact with proteases rather than being one.

UB is encoded by several genes in yeast (Ozkaynak et al. 1987). These genes are found in two basic forms in most organisms. Class I is a polyubiquitin gene which encodes an uninterrupted polyprotein of tandemly repeated UBs (UBI4) presumably released by post-translational mechanisms which cause cleavage at Gly-Met peptide bond that joins the repeats. Class II is a fusion between a single UB and of the other sequences, of either 52 (UBI1 and UBI2) or 76 amino acids (UBI3). UBI1 gene encodes a 128 residue protein consisting of UB followed by 52 predominantly basic residues, with the tail sequence which resembles

cysteine-containing DNA-binding motif of the *Xenopus* transcription factor TFIIIA (Miller et al. 1985). The fusion protein encoded by the UBI2 gene is identical to UBI1 gene product, despite a 15 percent divergence at the nucleotide sequence level between their coding regions. A TFII-A like putative DNA binding motif present in the UBI1 and UBI2 tails is also found in the UBI3 tail (Ozkaynak et al. 1987).

The expression of UB genes is tightly regulated. UBI1,2, and 3 in yeast are all expressed in cells during their exponential growth phase. While UBI3 is expressed under all conditions UBI1 and 2 are turned off during heat shock or cell starvation (Ozkaynak et al. 1987). UBI4 gene, which is expressed at low levels in cells during the exponential growth, is strongly induced by different stress conditions such as heat shock or starvation (Ozkaynak et al. 1987, Finley et al. 1987). The number of repeats per polyubiquitin locus varies considerably among and also apparently within species, suggesting that these loci often undergo unequal crossovers (Sharp and Li 1987).

1.3.1.2. Ubiquitin in Neurodegenerative Diseases

Ubiquitin is the common component which is found in diverse inclusion bodies in various neurodegenerative diseases, thus suggesting that UB may be the common link in the molecular pathology. Lewy bodies in Parkinson's disease, Pick bodies in Pick's disease, and NFTs and amyloid bodies in Alzheimer's disease are very well studied examples of this (Manetto et al. 1988, Lowe et al. 1988, Mori et al. 1987, Cole and Timiras 1987, Perry et al. 1987). Diffuse Lewy body disease also a common cause of dementia with the incidence of 15 to 27 percent of cases of dementia, show highly ubiquitinated Lewy bodies (Mayer et al. 1989a). Elevated levels of UB gene expression were detected in brain and spinal cord in motor neuron disease (Heggie

et al. 1989). Although antibodies against UB may react with microtubule of cultured cells (Magnusson and Wieloch 1989), UB is not usually found conjugated to the cytoskeletal proteins from which the inclusion bodies are derived. It has been shown that abnormal phosphorylation of τ occurs prior to its incorporation into PHF and leads to its accumulation in the nerve cell body and also that ubiquitin is seen associated only with a neurofibrillary tangle of PHF which is readily formed (Bancher et al. 1990, Bondareff et al. 1990). Thus it is suggested that UB accumulation is probably related to the process of neurodegeneration as a reflection of UB function in the proteolysis of abnormal or modified proteins, or could relate to the cellular stress before cell death. The molecule(s) which undergoes ubiquitination in AD brain is unknown, however the immunoreactivity of ubiquitin is removed after the removal of the fuzzy coat of paired helical filaments. Recently a MAP-related 30kD protein which is ubiquitinated and shows homology to τ was found, although its possible role in neurodegenerative pathology remains to be examined (Fried et al. 1990). Ubiquitin carboxy terminal hydrolase (PGP9.5) is also selectively present in ubiquitinated inclusion bodies in neurodegenerative diseases (Mayer et al. 1990). PGP9.5 studies may clarify the staging of ubiquitinated inclusion body biogenesis and provide a marker of the level of activity of the degenerative process.

Similar to senile plaques in the brains of AD patients, many Kuru plaques contain ubiquitin-positive immunoreactivity, suggesting that similar mechanisms are involved in neuritic changes in cerebral plaques in Creutzfeldt-Jakob and AD, despite differences of amyloid proteins in plaques (Suenaga et al. 1990a). Interestingly, PrP scrapie-associated fibrils are not covalently bound to ubiquitin.

Ubiquitinated inclusions are not confined to neurons but also occur in astrocytomas and, outside the nervous system in alcoholic liver disease (Mallory bodies), in myopathy (Lowe et al. 1988) and in some viral diseases (Mayer et al. 1989b).

1.4. Amyloidosis

Amyloidosis describes a heterogeneous collection of systemic diseases characterized by the extracellular deposition of proteinaceous amyloid fibrils of great chemical diversity in various organs and tissues of patients ranging clinically from arthritis to Alzheimer's disease (Glenner 1980, Cohen 1983, Kisilevsky 1987). In humans more than ten different amyloid proteins have been isolated and characterized. However in mice only three kinds of amyloids are known so far: AA, AS-sam, and SJL/J amyloid protein (Higuchi and Takada 1986).

All amyloids share a few common characteristics: 1) After congo-red staining, they appear as red-green birefringent material under polarized light (Bennhold 1922). 2) Because of their ß-pleated sheet structure (Eanes and Glenner 1968) all amyloids demonstrate common X-ray diffraction pattern. 3) All amyloids appear as unique 70-90 Å fibrillar structures with electron microscopy (Cohen and Calkins 1959, Cohen 1967). Virchow popularized the term "amyloid" in 1854 which has its root in the Latin-Amylum and Greek-Amylon. At that time based on the blue staining of these bodies with iodine, Virchow assumed that they are composed of cellulose or starch. However, Kekule and Friedreich discovered the protein nature of amyloid five years later (Friedreich and Kekule 1859).

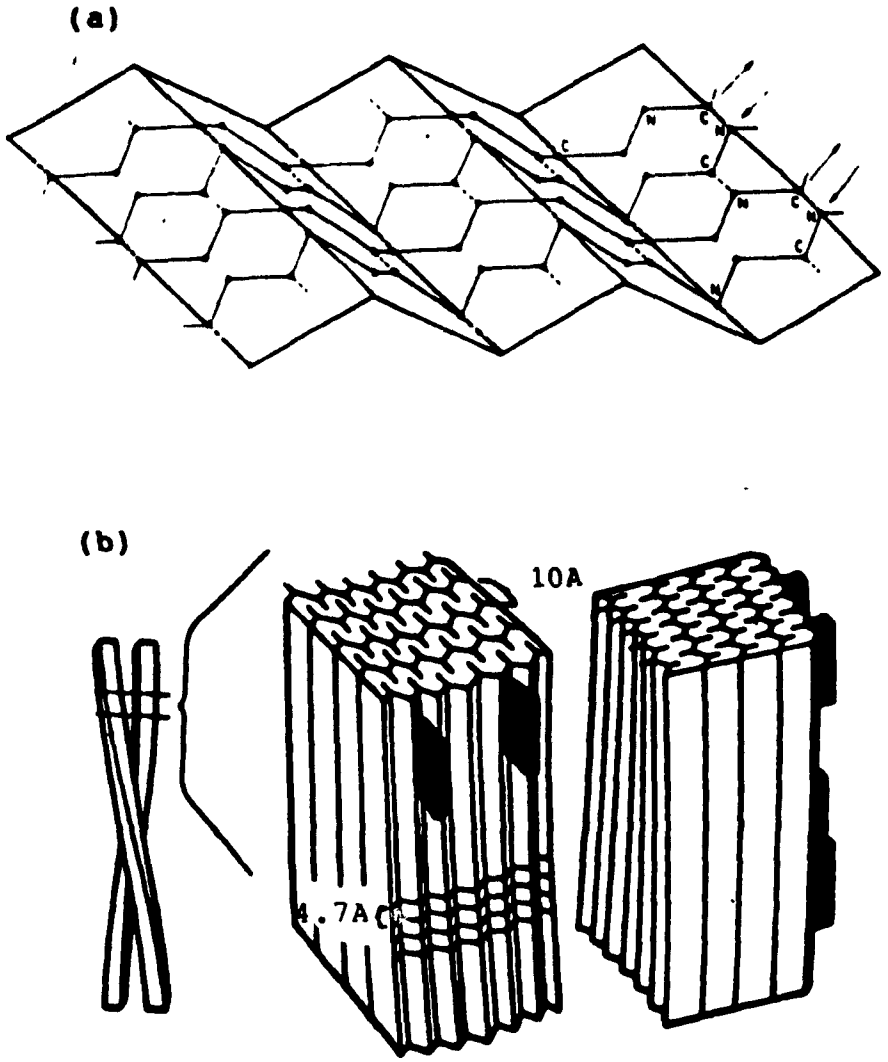


Fig.1.4. Presumed ß-pleated sheet secondary (a) and fibrillar tertiary (b) structure of amyloid proteins (Glenner 1980)

Although they dismissed carbohydrates completely, now we know about carbohydrate moieties such as proteoglycans or glycosaminoglycans linked to amyloid structures (Kisilevsky 1987).

Since the introduction of the experimental casein-induced amyloidosis (Kuczynski 1922), it has extensively been studied. AL type amyloid was the first to be sequenced. Glenner et al.

(1971a, 1971b) identified the light chain fragments as component of primary and myeloma amyloid. This was closely followed by sequencing the AA amyloid in secondary amyloidosis in animals and humans (Hermodsin et al. 1972, Levin et al. 1972, Skinner et al. 1974). Soon SAA or serum amyloid A, a major acute phase protein, was discovered (Levin et al. 1973, Bensson et al. 1975) and it was found that its expression is induced by interleukin-1 (Sipe et al. 1982).

Some primary amyloidoses are considered to be genetic disorders. For example in Familial Amyloid Polyneuropathy (FAP) fibrils demonstrate a single amino acid substitution of methionine for valine at position 30 of prealbumin gene localized on chromosome 18 (Tawara et al. 1984). A C to G mutation causing a single amino acid substitution (Gln instead of Glu) at position 22 of A4 amyloid protein is detected in Hereditary Cerebral Hemorrhage with amyloidosis of Dutch type (HCHWA-D), an autosomal dominant form of familial amyloidosis (Levy et al. 1990a & 1990b). Hereditary cerebral hemorrhage amyloidosis of Iceland type is another hereditary amyloid disease with mutations in gamma trace protein, cystatin-C (Cohen et al. 1983). Recently Frangione et al. (1990) copurified gelsolin amyloid (12kD) and A4 amyloid from Finnish Familial amyloidosis patient. Gelsolin amyloid presumably is the cleavage product of plasma gelsolin, a 93KD protein. By far the most common form of amyloidosis is found in AD (Kisilevsky 1987, Coleman 1990). Cerebral amyloid in AD, a 4kD protein also called A4 or ß-amyloid, which is also present in Down's syndrome (Masters et al. 1985a & 1985b), was sequenced by Glenner at al. (1984a & 1984b ,Wong et al. 1985) and Masters et al. (1985a). AD amyloidosis has been the focus of attention in the last few years.

1.4.1 Chemical Classification of Amyloid Proteins

1.4.1.1. A4/ß-Amyloid

This is the most common form of amyloid seen in Alzheimer's patients. It has 39 to 42 amino acid residues, and its approximate molecular size is 4kD (Glenner et al. 1984a, Masters et al. 1985a, Selkoe 1986). A4 amyloid is derived from a much larger precursor protein ranging in size from 110 to 130 kD (Selkoe 1988). This type of amyloidodosis is already discussed in more detail earlier in section 1.2.2.

1.4.1.2. AA Amyloid

AA amyloid, a 74 residue peptide with the approximate molecular size of 8.5kD, is generally seen during chronic idiopathic inflammatory conditions (Cohen 1983, Cohen and Skinner 1985, Kisilevsky 1987), or chronic bacterial infections (Cohen 1983, Mazur 1989). Familial Mediterranean Fever (FMF) is a hereditary disease associated with the systemic deposition of AA type amyloid protein (KIsilevsky 1983, 1987). AA amyloid in mice is derived from SAA2, one of the two serum amyloid A precursor proteins (Benditt et al. 1977). In humans, it is not clear yet which SAA isotype(s) is selectively deposited as AA amyloid protein. Six major SAA isoforms of human serum amyloid A protein exist which are assigned to three gene sequences (Strachan et al. 1989). For further information on AA amyloid see sections 1.1.6 and 1.4.3.

1.4.1.3. AL Amyloid

AL type amyloid was first sequenced by Glenner et al. (1971a). They also created AL amyloid fibrils from light chain of immunoglobulin under physiological conditions in vitro (Glenner et al. 1971b). AL amyloid is seen in some cases of multiple myeloma and immunoblastic lymphomas. It is a 12 to 18kD peptide which is primarily deposited in the kidneys. Most AL amyloids are

derived from lambda chain and have an isoelectric point (pI) of approximately 4.8. Multiple myeloma without AL amyloid is associated with monoclonal kappa chains with the molecular size of 15 to 51kD, and a pI value of 6.2 (Belliotti et al. 1990, Savage et al. 1989).

1.4.1.4. ß2-microglobulin Amyloid

Chronic hemodialysis is usually associated with ß2-microglobulin amyloid which primarily deposits as cystic bone lesions (Mikawa et al. 1990, Zingraff et al. 1990). ß2-microglobulin may also complicate chronic peritoneal dialysis (Brown et al 1990). Lysine specific cleavages of ß2-microglobulin amyloid have recently been reported (Linke et al. 1989).

1.4.1.5. Gelsolin Amyloid

This is a recently discovered amyloid seen in hereditary amyloidosis of Finnish type (Maury et al. 1990). Gelsolin amyloid varies in size from 9kD to 12kD, and is derived from a much larger (93kD) precursor protein, serum gelsolin, an actin modulating protein (Frangione et al. 1990).

1.4.1.6. Prion Amyloid

Prp27 amyloid which is derived from the larger (50kD) prion protein is seen in Kuru plaques of Scrapie and Creutzfeldt-Jacob disease. Intact scrapie prion protein is shown to aggregate and form amyloid like structures in vitro (Prusiner et al. 1983). Prp27 amyloid is also found in the brains of patients with Gerstsmann-Straussler syndrome, the only disease associated with a mutation in the prion protein gene, and transgenic mice carrying the scrapie prion gene develop scrapie and amyloid plaques (Scott et al. 1989). Trace amounts of A4 amyloid is also present as a contamonant in Kuru plaques. Ubiquitin is found to be associated with these plaques (Lowe et al.1990a).

1.4.1.7. Transthyretin Amyloid

Familial Amyloidotic Polyneuropathy (FAP) is an autosomal dominant disease associated with the systemic deposition of prealbumin or transthyrethin amyloid. A mutation (Val. to Met.) at position 30 is responsible for deposition of prealbumin as amyloid. Transgenic mice bearing the defect in transthyretin develop amyloid deposits (Shimada et al. 1989).

1.4.1.8. Islet Amyloid Polypeptide

Islet amyloid polypeptide (IAPP) is a recently discovered protein which is synthesized by normal islet ß cells and is secreted along with insulin from pancreas (Johnson et al. 1989). It opposes the action of insulin in peripheral tissues. IAPP which is composed of 89 amino acid residues is abnormally cleaved into the 39 residue islet amyloid (IA) peptide. IA deposition in pancreas contributes to the development of type II (non-insulin-dependent) diabetes melitus by destroying the ß cells secreting insulin (Nishi et al. 1989 & 1990).

1.4.1.9. Cystatin C Amyloid

Cystatin C amyloid is seen in Iceland hereditary congophilic angiopathy with cerebral hemorrhage which is an autosomal dominant disorder (Ghiso et al. 1986, Grubb et al. 1984). Cystatin C (gamma trace), a relatively small molecule (13kD), carries a mutation of glutamine to leucine at position 58 in Iceland hereditary amyloidosis (Ghiso et al. 1986) in which amyloid accumulates around cerebral blood vessels. The human cystatin C gene is located on chromosome 20 (Abrahamson et al. 1989).

1.4.2. Clinical Classification of Amyloidosis

Apart from chemical protein nature of amyloids, amyloidosis can be classified into three major clinical groups (Kisilevsky 1987): 1) Primary amyloidosis which is mainly caused by plasma cell dysfunction in multiple myeloma or other B-cell malignancies and appears denovo without any preceding disorders. 2) Secondary amyloidosis which results from existing chronic inflammatory disorders such as rheumatoid arthritis, ankylosing spondilitis and occasionally systemic Lupus erythematosus, tuberculosis, leprosy or cancer (Hiasa et al. 1983, Vanatta et al. 1983). 3) Familial amyloidoses which are considered hereditary or genetic disorders. This includes Familial Mediterranean Fever (FMF) among Isreali Jews associated with recurrent inflammation and polymorphonuclear leukocyte dysfunction (Schwabe and Lehman 1984, Meyerhoff 1980), and FAP among the Portuguese and Japanese. These three classes describe conditions in which many organs are involved in amyloidosis. 4) Isolated organ specific amyloidosis: although AD and DS were considered the most common organ specific amyloid diseases (Kisilevsky 1987), now there is evidence that A4 fibrils may also occur in other organs as well. Endocrine amyloidosis usually involves the specific gland secreting the amyloidogenic hormone. Islet amyloid is an example of organ specific amyloid which appears in the pancreas of patients suffering from non-insulin dependent type II diabetes (Nishi et al. 1990). Clinical classification has certain limitations. For example it does not provide any assurance that amyloid deposits within a group will indeed be uniform (Kisilevsky 1987).

1.4.3. Serum Amyloid A Protein

AA amyloid deposition occurs in two steps (Teilum 1952, Brandwein et al. 1985a & 1985b, Sipe et al. 1978, Sipe at al. 1986). Preamyloid phase is associated with elevation in serum acute phase proteins including serum amyloid A (SAA) (McAdam et al. 1978, Hoffman et al. 1984, Tape at al. 1988, Meek et al. 1989). SAA is the larger precursor protein for AA amyloid and exists as apolipoprotein in association with high density lipoprotein (HDL) (Benditt and Eriksen 1977, Benditt et al. 1979). SAA consists of 104 amino acid residues (Parmelee et al.1982, Sletton et al. 1983, Dwulet et al. 1988) with a molecular weight of approximately 12-14kD. It is not glycosylated, and is mainly synthesized by hepatocytes (Selinger et al. 1980) in response to inflammation and IL-1 (Sipe et al. 1979 & 1982, Ramadori et al. 1985). The biological function of SAA is unknown, although autocrine induction of collagenase gene by SAA-like proteins have recently been demonstrated (Brinckerhoff et al. 1989, Sack and Talbot 1989).

Following the proteolytic cleavage of the C-terminal fragment of SAA2 in mice and its removal, a smaller protein, AA amyloid, is generated (Husebek et al. 1985, Tape et al. 1988) which spontaneously undergoes fibril formation (Glenner 1980). Although a major portion of AA amyloid consists of 76 amino acids (Levin et al. 1972, Skinner et al. 1988b), its subspecies varying between 45 and 94 residues also exist (Levin et al. 1972, Sletton et al. 1976, Moyner et al. 1980, Westermark et al. 1987, 1989). Therefore, it appears that SAA cleavage occurs in variable sites. The major cleavage sites for SAA from both and humans and mice appear to be Ser-Leu and Try-Met bonds at positions 76 and 77, respectively (Ericsson et al. 1987, Hoffman et al. 1984). The presumed protease(s) responsible for this cleavage has so far eluded detection.

There are three genes and a pseudogene for SAA in Balb/c mice (Lowell et al. 1986, Stearman et al. 1986), however only two gene products, SAA1 and SAA2 are detected (McAdam and Sipe 1976, Meek et al. 1986). Although mRNA for SAA3 gene is detected in the liver, spleen, and other tissues of mice, its corresponding protein has not been identified in serum (Ramadori et al. 1985, Meek and Benditt 1986). SAA2 is the only precursor of AA fibril protein in mice and is selectively removed from the circulation (Baltz et al. 1986a & 1986b, Hoffman et al. 1984). The SAA gene family in humans codes for two major and four minor isotypes (Eriksen and Benditt 1980, Baysserman et al. 1980), and their complete amino acid sequence and genomic DNA sequences are yet to be resolved (Woo 1986).

1.4.4. Serum Amyloid P Protein

Amyloid P protein is composed of ten identical glycosylated subunits which are assembled as pentameric structures adjacent to amyloid fibrils (Pinteric et al. 1976, Osmond et al. 1977, Pepys et al. 1977). Their primary structure is identical to serum amyloid P (SAP) component (Skinner et al. 1980, Pepys et al. 1982, Mantzouranis et al. 1985, Prelli et al. 1985). SAP shares the overall homology of 66 percent with C-reactive protein (Pepys and Baltz 1983, Mantzouranis et al. 1985, Prelli et al. 1985, Woo et al. 1985). However the functional importance of this resemblance is unclear. It has been reported that SAP can modulate immune responses and is inhibitory to elastase like proteases (Li et al. 1984, Li and McAdam 1984). SAP binds to both AA-and AL-amyloids in the presence of calcium in vitro (Pepys et al. 1982). Amyloid deposits are rich in calcium (Kula et al. 1977) and this may account for the binding of SAP to amyloid fibrils in vivo (Baltz et al. 1986b & 1986b). Amyloid P has been detected in association with all forms of amyloid (Pepys et al. 1982).

1.4.5. Diagnosis and Treatment of Amyloidosis

The specific diagnosis of amyloidosis has been based on examining tissue biopsies sectioned and stained with the Congo red method (Bennhold 1922). Then combined with polarized microscopy, this method becomes the simplest and most useful histological test for the detection of amyloid deposits in tissues (Cohen and Skinner 1985). When a secondary underlying disorder or chronic inflammatory disease is present, amyloidosis is suspected. Biopsies can be obtained preferably from the rectal area, or other sites including the skin, gums or the suspected organs such as the kidneys and liver. In order to establish the relationship between AL amyloid and multiple myeloma, electrophoretic and immunoelectrophoretic studies on serum or urine should be performed when the biopsy is positive for amyloid (Cohen et al. 1983). A recent study has shown that radiolabelled SAP can be used as a probe for the diagnosis and monitoring of the extent of systemic amyloidosis (Hawkins et al. 1990).

In secondary amyloidosis, amyloid deposits get resorbed when the chronic inflammation is treated. However, systemic amyloidosis has a rather poor prognosis and progresses slowly leading to death in several years mainly because of renal failure (Cohen 1983). Although there is no specific therapy for amyloidosis, several agents with questionable efficacy have been used. Alkylating agents such as melphalan are used to block the synthesis of light chains, the precursor of AL amyloid, by plasma cells in multiple myeloma. However, they have serious side effects such as the development of acute leukaemia. There is evidence that immunosuppressive drugs and cytotoxic anti-inflammatory agents can improve the prognosis of rheumatoid arthritis or juvenile chronic arthritis which has been complicated by AA amyloidosis (Schnitzer and Ansell 1977, Berglund et al. 1987, Ahlmen et al. 1987). Colchicine has been shown to be effective in

preventing acute attacks in patients with FMF and AL-amyloidosis (Cohen and Connors 1987). Moreover the inhibitory effect of colchicine on amyloid deposition was shown in experimental mouse models (Brandwein et al. 1985a). The exact mechanism of action of colchicine is unknown, although it has been suggested that colchicine, like vinblastin, is an effective inhibitor of cellular protein degradation probably acting on microtubuli involved in the lysosomal pathway (Grinde and Seglen 1981).

Dimethylsulfoxide (DMSO) is another candidate drug under investigation for the treatment of amyloidosis (Cohen 1983). Anti-inflammatory activity of prostaglandin E1 in preventing amyloid deposition in CBA/J mice has also been reported (Brandwein et al. 1985b). PGE1 which apparently blocks AEF formation, may provide us with the therapeutic means for amyloidosis.

1.4.6. Amyloidogenic Factors

Amyloidosis can be induced in mice experimentally by injection of dead or living bacteria, or bacterial lipopolysaccharide (Waalen et al. 1980), silver nitrate (Higguchi and Takeda 1986), casein or azocasein (Kuczynski 1922), gelatin or egg protein (Letterer 1926), phenolic solutions (Letterer 1934), tar (Twort and Twort 1932), tumor grafting (Lubarrsch 1897), *cysticercus* infection (Benassi and Baggi 1952), *Echinococcus* infection (Ali-Khan et al. 1983b). This suggests the existence of a common mechanism of action to such basically dissimilar agents. The ability of mice to develop amyloid by exposure to electrical shock (Hall et al. 1960) or the occurrence of amyloidosis in fighting mice (Page and Glenner 1972) or in fighting Pekin ducks (Cowan et al. 1970) suggests that the basic underlying disturbance common in all amyloid diseases might be the induction of the stress condition. Since aging may be considered a somatic sequela to chronic stress, it may help explain the prevalence of amyloidosis in aging mice (Hall et al. 1960, Yokota et al. 1989b).

Amylodosis may occur spontaneously in different animals such as mice, cats, dogs and ducks (Page and Glenner 1972, Dibartola et al. 1985, Gorevic et al. 1977). Spontaneous age-related or senile amyloid in mice (AS-sam) is distinct from AA amyloid which is spontaneously deposited in SJL mice (Scheinberg et al. 1976) and SAM-P mice (Matsumura et al. 1982).

1.4.7. Accelerated Amyloidosis

Teilum in 1952 suggested the two phase theory of pathogenesis in experimentally induced amyloidosis. He hypothesized that amyloid deposition occurs in two steps. The preamyloid phase which usually lasts for a number weeks and is followed by amyloid deposition in susceptible organs. In 1966, Werdlein and Ranlov, based on the assumption that autoimmunity may be involved in amyloid formation, showed that intraperitoneal transfer of live spleen cells from amyloidotic animals into normal animals could cause amyloid deposition in recipient mice. Since dead cells as well as their extracts, when given along with an inflammatory stimulus were also amyloidogenic, they used the term "amyloid transfer factor". They and others also showed that normal spleens contained residual amyloid transfer factor activity (Varga et al. 1986, Axelrad et al. 1982, Abankwa and Ali-Khan 1988), and this activity increases dramatically in normal aging mice (Yokota et al. 1989b) and in preamyloid tissue (Axelrad et al. 1982, Abankwa and Ali-Khan 1988, Varga et al. 1986). Unlike casein-induced experimental amyloidosis, amyloid deposition in the accelerated model occurred shortly (24 to 48 hr) after administration of the crude splenic extract. Later on the name was changed to "amyloid accelerating factor" and "amyloid enhancing factor" (Axelrad et al. 1975, Axelrad and Kisilevsky 1980, Axelrad et al. 1982). A concomitant inflammatory stimulus such as nitrogen mustard (Hardt and Ranlov 1973), Casein (Hardt and ranlov 1973, Axelrad et al. 1975) or silver nitrate (Abankwa and Ali-Khan

1988) is needed for this biological action, since AEF by itself is not amyloidogenic (Varga et al. 1986, Ali-Khan et al. 1988, Axelrad et al. 1982, Kisilevsky et al. 1977, Abankwa and Ali-Khan 1988b, Alizadeh-Khiavi and Ali-Khan 1988, Kisilevsky et al. 1986, Yokota et al. 1989a). The percentage of the macrophages, neutrophils, mast cells and lymphocytes containing cytosolic AEF increases significantly in amyloidotic mice (Alizadeh-Khiavi and Ali-Khan 1988), and neutrophil (Kisilevsky et al. 1977, Yokota 1989a). Macrophage and lymphocyte extracts or mitogen-stimulated spleen cell supernatants prepared from amyloidotic, but not from normal mice, contain significant levels of AEF activity (Abankwa and Ali-Khan 1988b). AEF level is also increased in aging mice (Yokota et al. 1989b). Shirahama et al. (1990) demonstrated that AEF-rich macrophages are capable of processing SAA to AA amyloid fibrils. AEF activity is associated with both cytoplasmic and nuclear fractions of cells harvested from amyloidotic mice (Hardt and Hellung-Larsen 1972). Although it was first presumed that AEF is a glycoprotein (Axelrad et al. 1982) the biochemical nature and its identity have remained unclear until now.

AEF activity is ubiquitously distributed in AEF protein fractions ranging from 5 to 1000kD in size, suggestive of the self-aggregation or conjugation of AEF to other molecules (Ali-Khan et al. 1988, Axelrad et al. 1982, Abankwa and Ali-Khan 1988b, Alizadeh-Khiavi and Ali-Khan 1988, Yokota et al. 1989a, Niewold et al. 1986 & 1987). It has been suggested that AEF might be glycosaminoglaycans (Kisilevsky 1987), or fibronectin (Kawahara et al. 1989). The in vivo bioactivity of both crude and murine AEF is abolished with phenyl-methyl sulphonyl fluoride, suggesting a serine-protease nature for AEF (Ali-Khan et al. 1988, Abankwa and Ali-Khan 1988b, Alizadeh-Khiavi and Ali-Khan 1988). Although AEF activity is mainly studied in CBA/J (Axelrad et al. 1982) and C57 BL/6 strains of mice (Abankwa and Ali-Khan 1988a & 1988b, Alizadeh-Khiavi and Ali-Khan 1988), it is not restricted to these particular strains. AEF

has also been demonstrated to cause accelerated amyloidosis in A/J mice (Wohlgetthan and Cathcart 1980), a strain known for its resistance to amyloid induction (Wholgethan and Cathcart 1979). AEF passive transfer into A/J mice significantly reduces the induction period for AA amyloidosis from months to days, emphasizing the key role that AEF might play in amyloid deposition (Wholgethan and Cathcart 1980). Human AEF extracted from brain and spleen samples is transferable to mice (Varga et al. 1986, Ali-Khan et al. 1988, Keizman et al. 1972). AEF has also been demonstrated in hamsters with AA amyloid (Hol et al. 1985 & 1986). It has been extracted from patients with familial amyloid polyneuropathy and patients with AA and AL type amyloidosis (Varga et al. 1986, Kisilevsky 1987). This functional conservation among different species suggests that AEF is possibly a structurally conserved molecule throughout the biological evolution (Alizadeh-Khiavi and Ali-Khan 1990, Robitaile et al. 1990, Alizadeh-Khaivi et al. 1990a, 1990b, Chronopoulos et al. 1990a, 1990b).

Therefore, AEF appears to be the common pathogenetic link in diverse forms of amyloid-related disorders. AEF is essential in AA amyloid fibril formation (Kisilevsky 1983, Brandwein et al. 1985a & 1985b, Deal et al. 1982).

My main research interest has been to purify AEF and investigate its behaviour in amyloidosis. The present experiments were carried out in our well-characterized alveolar hydatid cyst (AHC)-mouse model of inflammation-associated AA amyloidosis (Ali-Khan et al. 1983b, Alkarmi and Ali-Khan 1984, Alkarmi et al. 1986, Treves and Ali-Khan 1984, Du and Ali-Khan 1990). Brain autopsy samples from AD patients were also used in these studies (Alizadeh-Khiavi et al. 1990a). Based on the partial amino acid sequencing data, immunochemical and functional criteria, I have recently identified AEF activity in ubiquitin, isolated in non-denaturing condition, from murine amyloidotic tissues (Alizadeh-Khiavi et al. 1990b), as well as from AD brain extracts (Alizadeh-Khiavi et al. 1990a).

MATERIALS AND METHODS

2.1. Reagents

The following chemicals were used: 2-mercaptoethanol from Kodak (USA), acrylamide, N-N' methylene bisacrylamide, sodium dodecyl sulfate (SDS), CNBr-activated Sepharose 4B gel, Sephacryl S-200 HR, DEAE-Sepharose CL-6B, and CM-Sepharose from Pharmacia (Canada), Coomassie blue R-250, goat anti-rabbit IgG conjugated to alkaline phosphatase, silver staining kit, Mini-Protein II electrophoresis apparatus and Model 22 Electro-Eluter from Bio-Rad (Canada), silver nitrate, protease-peptone, Hank's balanced salt, fetal calf serum, heparin-sodium salt, bovine ubiquitin (lot. No. 106F-9355), goat anti-rabbit IgG [F(ab')2 fragment] conjugated to fluorescein-isothiocyanate, azocasein (lot no. 106F-8015), human hemoglobin, bovine serum albumin, polyethylene glycol (PEG) compound (mol. wt. 15,000-20,000), Congo red, ethanolamine, diethanolamine, Freund's complete and incomplete adjuvant, *p*-phenylenediamine, *p*-nitrophenyl phosphate disodium, Tris (hydroxymethyl) aminomethane, monobasic and dibasic sodium phosphate, sodium chloride, calcium chloride, and Triton X-100 from Sigma Chemical (Mo. U.S.A.), *S. enteritidis* lipopolysaccharide, RPMI-1640, L-glutamate, Streptomycine-penicillin (100X) and HEPES (100X) from Difco-BRL (USA), keyhole limpet hemocyanin (KLH) and calf thymus ubiquitin (lot no. 701799) from Calbiochem, 6 to 8 week-old male C57BL/6J mice from Charles River (Montreal, Canada), ELISA Immulon 2 flat bottom microtitration plates from Dynatech Laboratories (Virginia, U.S.A.), and S & S NC Nitro-cellulose paper from Schleicher & Schuell (USA), 15nm gold-strepavidin from Amersham (Canada).

2.2. Infection and Preparation of Crude AEF

The strain of larval *Echinococcus multilocularis* (alveolar hydatid cyst, AHC), its maintenance in mice, the methods of isolation of AHC and its inoculation in mice have been reported previously (Ali-Khan 1978, Ali-Khan et al. 1983a, Du and Ali-Khan 1990). Male C57BL/6 mice, 6-8 weeks old, were infected intraperitoneally (i.p.) with 250 AHC. These mice develop multi-organ amyloid (AA-type) deposits in one week post-infection (p.i.) (Du and Ali-Khan 1990). In the present experiments, the AHC-infected mice were sacrificed at 8 weeks p.i. and their spleens and livers were homogenized in cold 100 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS; 1 g tissue/10ml buffer; 3 min, Tekmar Tissuemizer, Ohio, U.S.A.). The tissue suspension was stirred (60 min; 4°C), centrifuged (30,000 g, 60 min, 4°C) and the supernatant used as crude AEF (Alizadeh-Khiavi et al. 1990b, Alizadeh-Khiavi and Ali-Khan 1988). Crude AEF was also extracted in a similar fashion from -80°C frozen AD brain tissues obtained from Douglas Hospital Research Center, Brain Tissue Bank, Verdun, Quebec (Alizadeh-Khiavi et al. 1990a).

2.3. Protein Determination

Protein concentrations were determined using the Bio-Rad protein assay kit following the manufacturer's instructions (Bio-Rad, Toronto, Canda). Bovine serum albumin was used as the standard.

2.4. Identification of AEF Activity in a Low Molecular Weight Protein from Mouse

The pH of the crude AEF was adjusted to 5 with 1M phosphoric acid. The turbid suspension was stirred (45 min, 4°C), centrifuged (30,000 g, 60 min, 4°C) and the supernatant was dialyzed against distilled water (Spectrapor; mol. wt. cut-off 3.5K; 4°C), concentrated against polyethylene glycol (PEG) and lyophilized. The lyophilized sample (300 mg) was dissolved in 3 ml of 25 mM

Tris-HCl, pH 8.0 and applied to a Sephacryl S-200 HR column (1.5 X 85 cm; flow rate 12 ml/hr) pre-equilibrated with the above buffer. As indicated (Fig. 3.1), the F1 to F4 fractions were collected, concentrated against PEG, dialyzed against PBS, then assayed (0.2 mg protein/mouse) for AEF activity (Abankwa and Ali-Khan 1988a & 1988b, Alizadeh-Khiavi and Ali-Khan 1988). The activity co-fractionated with F1 and the small F2 fractions (Fig. 3.1). In SDS-PAGE (Laemmli 1970, see below), the F1 demonstrated numerous peptides, therefore we chose F2 which had 5 prominent low molecular weight peptides for electro-elution and AEF bioassay. F2 (2 mg protein) was mixed with 400 μl of 2X Laemmli's sample buffer (Laemmli 1970), and instead of boiling which destroys AEF activity (Axelrad and Kisilevsky 1980), the mixture was incubated for 2 hr at 37°C, electrophoresed and the gel stained. The Coomassie stained bands from F2 were excised and electro-eluted using 50 mM ammonium acetate containing 0.1% SDS. The fractions EP-AEF-a to EP-AEF-c (Fig. 3.1, Table 3.1) were exhaustively dialyzed (Spectrapore, mol. wt. cut-off 3.5K) against PBS. The protein concentration of the Coomassie-stain tinged electro-eluted a to c fractions was adjusted to approximately 100 μg/ml with PBS at 595 nm before determining their AEF potency (1 ml/mouse).

2.5. Amino Acid Sequence Analysis

Since EP-AEF-a was the only AEF positive electroeluted protein in the F2 fraction, it was submitted to repetitive Edman degradation in an ABI Model 470 peptide microsequencer, and the phenylthiohydantoin (PTH)-derivatives of the released amino acids were analyzed on-line with ABI model 120A PTH-amino acid analyzer (Dr. M. Van der Rest, Center for Human Genetics, Shriner's Hospital, Montreal, Quebec). The partial amino acid sequence of the AD-brain derived ubiquitin was also determined using this technique.

2.6. Large Scale Purification of Ubiquitin from Amyloidotic Murine or AD Tissues

Large scale purification of AEF was carried out following the methods developed for the purification of ubiquitin from bovine erythrocytes (Matsumoto et al. 1984) with minor modifications. Briefly, the crude AEF extract from murine liver and spleen was diluted (4.5:1) with chilled 95% ethanol-chloroform (1:1; -20°C). The clarified supernatant was dialyzed (Spectrapor membrane, mol. wt. cut off 3.5K) against distilled water and lyophilized. The lyophilized material was dissolved in 25mM Tris-HCl, pH.8 (T-buffer) and passed through a DEAE-Sepharose CL-6B column pre-equilibrated with T-buffer. The gel bound proteins were eluted by a linear gradient of 0 to 100mM NaCl in T-buffer. The flow through and the initial NaCl gradient eluted fractions containing monomeric ubiquitin (determined by SDS-PAGE and Western immunoblotting) were pooled, and subjected to gel filtration (Sephacryl S-200 HR column; 2.6 x 93 cm; flow rate 15 ml/hr; pre-equilibrated with T-buffer). The fractions containing almost all the monomeric ubiquitin were pooled, dialyzed against 50mM ammonium acetate, pH.5 and passed through a CM-Sepharose column (pre-equilibrated in the dialyzing buffer). After the absorbance of the flow through at 280 nm reached the base line, the bound homogeneous ubiquitin was eluted with 50mM ammonium acetate, pH 6.4.

Frozen (-80°C) coronal brain sections (18.6 g) from 3 Alzheimer's disease patients (male, 67 yrs, female, 86 yrs and male, 79 yrs) were homogenized in 20 mM Tris-HCl, 1mM EDTA, 5 mM 2-ME, 150mM NaCl, pH 7.5 (10 ml/g) and centrifuged (30,000 g, 60 minutes, 4°C). One half of the supernatant was treated with heat (90°C, 20 minutes) (Alizadeh-Khiavi et al. 1990a, Haas and Wilkinson 1985) and the other half diluted (4.5:1) with 95% ethanol-chloroform (1:1;-20°C) (Matsumoto et al. 1984). The clarified supernatants were dialyzed (Spectrapor membrane, mol wt cut-off 3.5kD) against distilled water and lyophilized. Each sample was treated similarly in the subsequent purification steps.

2.7. Recombinant Proteins

Recombinant wild type and mutagenized ubiquitin samples were kindly provided by Dr. Victor Fried. Details of the DNA synthesis and assembly is described somewhere else (Ecker et al. 1987). All the mutations were introduced into the synthetic ubiquitin gene by replacing parts of the gene with synthetic DNA encoding the desired mutation. Gene expression in *E. coli* was under the control of λP_L promoter. Bacterial cells were lysed with EDTA and lysozyme (Ecker et al. 1987), and then ubiquitin species were purified from the extract (Fried et al. 1987).

2.8. Mouse Bioassay for Determination of Amyloid Enhancing Factor (AEF) Activity

This was carried out as described (Ali-Khan et al. 1988b, Axelrad et al. 1982). Briefly, crude AEF or its various fractions, with or without 10mM $CaCl_2$, were injected i.p to mice. In the initial experiments, each mouse also received four daily subcutaneous (s.c) injections of 0.5 ml of 1% $AgNO_3$ and sacrificed 24hr after the last $AgNO_3$ injection (Ali-Khan et al. 1988b); multiple inflammatory stimuli have also been used by others (Varga et al. 1986, Niewold et al.1986 & 1987, Yokota et al. 1989b). In subsequent assays the inflammatory stimulus consisted of one s.c. injection with 0.5 ml of 2% $AgNO_3$; mice were sacrificed after 48 or 72 hr (Axelrad et al. 1982). The spleens were sectioned (8μm), stained with alkaline Congo red and examined with polarized light to identify congophilic green birefringent amyloid deposits in the splenic perifollicular areas (PFA). Amyloid deposition was graded between ± to 3+ depending upon the approximate PFA circumferential area covered with amyloid: ±: less than 10%; 1+: 10-25%; 2+: 25-50%; 3+: 50-100%. Several controls were used in these experiments: groups of mice received i.p. injection of either crude AEF or tissue derived monomeric ubiquitin only, PBS with 10 mM $CaCl_2$ i.p and one (2%) or four (1%) s.c. injections with $AgNO_3$; bovine serum albumin (10 mg/mouse, i.p), human hemoglobin (10 mg/mouse, i.p) or azocasein (100 μg/mouse, intravenously) and four daily s.c. injections with 0.5 ml of 1% $AgNO_3$. Spleen sections from the control mice were processed as described.

AEF activity of recombinant ubiquitin species was assayed as described above. Briefly C57BL/6 mice received an i.p. injection of 50μg ubiquitin suspended in 0.5 ml of PBS and 10 mM $CaCl_2$ in conjunction with either one s.c. injection of 0.5 ml of 2% $AgNO_3$ or three daily s.c injections of 0.5 ml of 1% $AgNO_3$. Mice in the 2% $AgNO_3$ group were sacrificed at 48 h, and 24 h after the last $AgNO_3$ injection in the 1% $AgNO_3$ group. Their spleen sections were stained with Congo red and graded for AA deposits. Control mice received either i.p. 50 μg/mouse recombinant ubiquitin samples (Rc-WT UB; Table 3.6: group 8) only, or azocasein i.p. (50 and 100 μg/mouse; Table 3.6; groups 10 and 11), or PBS plus 10 mM $CaCl_2$ (0.5 ml; i.p.) (group 9) along with 4 daily s.c injections with 0.5 ml of 1% $AgNO_3$ or one single 2% s.c. injection of $AgNO_3$. These mice were sacrificed 48 or 96 hr later and their spleen sections were processed as previously described.

2.9. Electrophoresis and Immunoblotting

5 to 15 μg of various fractions were subjected to SDS-electrophoresis on 15 percent polyacrylamide gels (Laemmli 1970). Protein bands were electro-transferred onto nitro cellulose paper overnight at 0.3 mA constant current. Western analysis (Towbin et al. 1979) was performed using rabbit anti-ubiquitin IgG antibody (RABU) or anti-AA IgG antibody, and goat anti-rabbit IgG conjugated to alkaline phosphatase; N.C. paper was heated to 121°C for 20 min (by autoclaving; paper immersed in distilled water between two blotting papers) to enhance the immunoreactivity of ubiquitin and its adducts to RABU (Haas and Bright 1985). Alternatively ^{125}I-labelled donkey anti-rabbit Fab2 was used as the secondary antibody combined with autoradiography according to Amersham (Canada). Specificity of the immunoblotting was determined with RABU or RAA absorbed with bovine ubiquitin or purified AA. Alkaline polyacrylamide gel electrophoresis was performed under nondenaturing condition, without SDS or mercaptoethanol, in 8 percent gels using Mini Protein II apparatus from Bio-Rad (Canada) (Hames 1986).

2.10. Generation of Anti-AA and Anti-Ubiquitin Antibodies

Rabbit anti-bovine ubiquitin antibody (RABU) was produced as described before with minor modifications (Haas and Bright 1985, Alizadeh-Khiavi et al. 1990a & 1990b). Briefly, bovine ubiquitin (BU) cross-linked to keyhole limpet hemocyanin with 80 μl of 3% glutaraldehyde was used as the antigen (1:1 with Freund's complete adjuvant) to immunize rabbits. Three weekly boosters were given to the rabbits and one week after the last boost, their blood was collected. The antiserum was incubated (overnight, 4°C) with CNBr-activated Sepharose 4B conjugated to BU (5 mg protein/ml gel) and the bound protein was eluted from the gel with 0.1M glycine-HCl, pH2.8 containing 0.5M NaCl. The eluted protein was dialyzed against 0.1M sodium phosphate buffer pH7.4, containing 0.5M NaCl and passed through a Protein A-Sephrose gel column to elute RABU. RABU was dialyzed against PBS and its protein concentrations determined as described above. RABU was a high-affinity antibody as judged by immunocytochemical and immunoblotting criteria (Alizadeh-Khiavi et al. 1990a & 1990b, Chronopoulos et al. 1990a & 1990b). In some experiments, parallel immunoblots were prepared using another anti-ubiquitin antibody (Haas and Bright 1985), kindly provided by Dr. A.L. Haas, Department of Biochemistry. Medical College of Wisconsin, Milwaukie, Wisconsin, U.S.A.

Purified AA amyloid (Alkarmi et al. 1986) was used to immunize rabbit. The antiserum was passed through a Protein A-Sepharose gel column and rabbit anti-AA amyloid IgG antibody (RAA) was eluted following the above-mentioned procedure.

For control experiments these antibodies were absorbed, overnight at 4°C, with their respective antigens in the following proportions: 5 mg of BU with 0.2 mg/ml of RABU or 20 μg purified mouse AA amyloid with 4 μg/ml of RAA. The absorbed antisera were microfuged for 15 minutes at 4°C and the supernatants used for assessing the specificity of the immunostaining reactions.

2.11. Immunoaffinity Purification of Murine Ubiquitin and Some of Its Adducts

Crude mouse AEF (2 ml; total protein 8 mg) was incubated (overnight, 4°C) with 7 ml of CNBr-activated Sepharose 4B (Pharmacia, Canada) conjugated to 75 mg RABU (conjugation of RABU to the gel was carried out according to the manufacturer's instruction, Pharmacia). After exhaustive washing with 10mM phosphate buffer, pH 7.4 containing 0.5 M NaCl, bound proteins were eluted with 0.1 M glycine-HCl buffer pH 2.8 containing 0.5 M NaCl. The pH of the eluate was neutralized immediately with 1.7M Tris-HCl, pH 8.5. After dialysis against PBS the eluted material was concentrated against PEG and bioassayed for AEF. The specificity of the immunoaffinity purification method was confirmed by incubating crude AEF with 1M ethanolamine treated CNBr-activated Sepharose gel, without attached ligand, in similar proportions as above. Since only the eluate from RABU-bound gel contained protein components with AEF activity, it was fractionated on a Sephacryl S-200 HR column (1.5 x 85 cm; flow rate 10 ml/hr) pre-equilibrated with PBS. Two fractions, the first in the void volume (P1) and the other as a retarded fraction (P2) were obtained. They were concentrated against PEG, dialyzed against PBS and analyzed by electrophoresis (15% SDS-PAGE), immunoblotting with RABU, and the assay for AEF activity.

2.12. Neutralization of AEF Activity by Anti-Ubiquitin Antibody

Five groups of C57BL/6 mice received i.p. either different doses of RABU (0.4, 2.0, 5.0, and 10.0 mg/mouse), or 5.0 mg/mouse normal rabbit IgG. After 24 hr, mice were challenged with i.p. injection of crude AEF (0.2 mg) and one s.c. injection with 0.5 ml of 2% $AgNO_3$. In another neutralization experiment crude AEF (0.2 mg) was incubated (overnight, 4°C) with 0.1, 1.0, 2.0, and 4.0 mg of RABU or 4 mg of normal rabbit IgG and the mixture injected i.p. into mice in conjunction with one s.c. injection of 0.5 ml of 2% $AgNO_3$. Mice in each group were sacrificed 48 hr after the $AgNO_3$ injection and their spleen sections examined for amyloid deposits.

The above mentioned criteria were used for the selection of RABU dosage and the assay method for the neutralization of AEF activity in recombinant wild type ubiquitin (Rc-WT UB) (Alizadeh-Khiavi et al. 1990b). Briefly 100 μg of Rc-WT UB was incubated overnight with 4 mg RABU or 4 mg normal rabbit IgG. These samples were centrifuged at 14,000 g (30 min, 4°C) and the supernatants injected i.p. into two groups of 6 to 8 weeks old male, C57BL/6 mice in conjunction with one s.c. injection with 0.5 ml of 2% $AgNO_3$. Mice in another group received 100 μg Rc-WT UB only. All mice were sacrificed 48 hr later and their spleen sections graded for AA amyloid.

2.13. Immunohistochemistry

This was performed by strepavidin-biotin-peroxidase method as previously described (Alizadeh-Khiavi et al. 1990a). Briefly the cultured peritoneal cells or cryostat sections of spleens from mice already inoculated with ubiquitin and silver nitrate were fixed in acetone for 10 minutes at room temperature or deparaffinized in xylene for 20 minutes. Slides were air dried and permeabilized in 20 mM tris buffer pH 7.5 containing 0.15 M NaCl and 2% Triton X-100 for 10 min. After 3 X 5 min washes in TTBS (20 mM tris buffer pH 7.5 containing 0.15 M NaCl aqnd 0.2 percent tween 20), endogenous peroxidase was quenched in 3.3 percent hydrogen peroxide solution in methanol for 30 min at room temperature. Subsequently, slides were rehydrated sequentially in 100, 50, and 30 percent ethanol (2 min each), and after washing in TTBS, they were blocked in 10 percent normal horse serum (NHS) for 30 min at room temperature. Rabbit anti-AA IgG (1:800 dilution; 2 hr incubation) and rabbit anti-ubiquitin IgG (1:640 dilution; overnight incubation) were used as primary antibodies. Secondary biotinylated donkey anti-rabbit IgG (1:100 dilution; 30 min), Strepavidin horse radish peroxidase (1:100 dilution; 30 min) combined with a color developing substrate (0.2 mg/ml DAB in 0.5 M tris

buffer pH 7.6 containing 0.002 percent hydrogen peroxide were used for detecting the appropriate antigens in the tissue or cells. Hematoxyline was used as a counterstain and after dehydration in 30, 50, and 100 percent ethanol solutions, and 10 min incubation in xylene or xylene substitute slides were mounted in Permount. Cultured peritoneal cells were also stained with thioflavin S and examined under fluorescent microscope for amyloid detection as described elsewhere (Vasser and Culling 1959).

2.14. Immunoelectron Microscopic Localization of Ubiquitin and Amyloid-AA

Murine spleens from normal and 7 day 250 AHC post infected mice, positive for AA deposits, were treated as previously described (Linke et al. 1989b) with some modifications. Spleens were cut into small 1-2 mm pieces and fixed by immersion in 4 percent glutaraldehyde solution in PBS for 2 hr, then washed 4 times with PBS. The tissues were treated in 1 percent osmium in PBS for 1 hr in ice and washed 3 times (5 min) in PBS, and dehydrated with an increasing concentration of ethanol (25, 50, 75, 100 percent; 5 min each in ice). Spurr (LR White, Dolanon Instruments, Inc.; Hatfield, Pennsylvania) was used as the embedding medium and was polymerized for 10 hr at 70°C. 80 nm thick sections were cut using a diamond knife, and mounted on Formvar coated nickel grids. The grids were etched in 10% hydrogen peroxide for 10 minutes, washed in TTBS 3 times, blocked in 10% normal horse serum in TTBS, and incubated for 2hrs with RAA (1:200 to 1:800 dilutions; original concentration 10 mg/ml), or overnight with RABU (1:10 to 1:100 dilutions; original concentration 0.3 mg/ml), or with preabsorbed RAA or RABU. Grids were washed 5 times in TTBS and were subsequently incubated with biotinylated donkey anti-rabbit F(ab)2 (1:30 dilution; 1hr) and 15nm gold-strepavidin (1:20; 1hr). Uranyl acetate (4% alcoholic solution; 3 minutes) and Reynold's lead citrate (30 sec) were used for counterstaining the grids at room temperature.

Peritoneal cells from the same mice were obtained in Hank's balanced salt solution (HBSS) containing heparin (10 U/ml), washed 3 times in HBSS. PBS was used for the final wash and cells were lightly centrifuged (400 g; 10 min) to form a pellet which was treated as mentioned above.

2.15. Determination of SAA

Groups of C57BL/6 mice (3/group) received either $AgNO_3$ (one s.c. injection of 0.5 ml of 1% $AgNO_3$) only, ubiquitin (one i.p. injection, 10 mg/mouse, dissolved in PBS with added 10 mM $CaCl_2$) or crude AEF (one i.p. injection, 0.2 mg/mouse). Sera were collected after 16 hr and assayed for SAA concentrations by ELISA as described (Zuckerman and Suprenant 1986), using 96 well microtitration plates. Purified mouse AA amyloid (Alkarmi et al. 1986), solubilized in 0.1 M $NaHCO_3$ buffer, pH 9.6 was used (1.25 μg/ml, starting concentration) to develop the standard AA protein curve. Serial dilutions (1:2) of the standard AA protein and the sera (1:40, starting dilution) were incubated overnight at 4°C. Plates were washed in PT buffer (1.59mM KH_2PO_4, 8.4 mM Na_2HP0_4, 2.68 mM KCl, 137mM NaCl, 0.1% Tween 20) and treated sequentially with RAA (1:500), goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (1:1000, 60 min, 37°C), and the color reaction was developed (30 min, room temp) with *p*-nitrophenyl phosphate disodium (0.5 mg/ml) solubilized in diethanolamine (1M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8). SAA concentrations were calculated using Microplate Manager Data Analysis software (Bio-Rad, Toronto, Canada) and are expressed here as micrograms of SAA extrapolated from a standard curve developed with mouse AA amyloid.

2.16. Preparation of Peritoneal Exudate Cells

This was performed as described elsewhere (Abankwa and Ali-Khan 1988a, Shirahama et al. 1990) with some modifications, under aseptic conditions. Briefly, peritoneal cells from normal or 4day, 7day, or 3 weeks 250-AHC post infected C57BL/6 mice were harvested with HBSS containing heparin (10 U/ml), and washed 3 times in HBSS (250g; 15 min). RPMI-1640

supplemented with 300 mg/l L-glutamate, 10 mM HEPES, 100 mg/ml streptomycine, 100 U/ml penicillin and 10% bovine calf serum was used for the final wash. These cells were subsequently cultured in Rose chambers.

2.17. Indirect Immunofluorescent Staining (IFA)

Harvested peritoneal cells were cytocentrifuged onto glass slides and stained by IFA as described (Chronopoulos et al. 1990a). Briefly, the slides were fixed in ethanol (-20°C, 20 min), permeabilized in 2% Triton X-100 (5 min), washed and then exposed to 1:20 dilution of RABU (15 μg protein/ml) overnight at 4°C. The secondary antibody was goat-anti-rabbit IgG F(ab)2-conjugated to FITC (1:40). PBS containing 5% fetal calf serum, pH 7.4, was used in all the washing steps and for the dilution of the primary and the secondary antibodies. The specificity of the staining reaction was determined by exposing the cells to the preimmune rabbit serum (1:5), or RABU absorbed with bovine ubiquitin (5 mg of ubiquitin /0.2 mg of RABU; overnight; 4°C), and the conjugate. The slides were washed, stained with Evans blue (10 sec), mounted in PBS-glycerol (1:9) containing *p*-phenylenediamine (10 mg/ml) and examined under a Dialux-20 fluorescent microscope. At least 200 leukocytes from duplicate samples were examined under 40 x or oil immersion objective; nuclear morphology was used for leukocyte identification.

2.18. SAA Purification

Mouse serum SAA-HDL was isolated as described before (Eriksen and Benditt 1980). Briefly, C57BL/6 mice received subcutaneous injection of 0.5 ml of 2% silver nitrate solution; their sera were collected 16 hours later, brought to the density value of 1.063 with KBr and ultracentrifuged in 60Ti rotor for 20 hrs at 50,000 rpm. The density of the fractions containing SAA, as determined by SDS-PAGE and Western immunoblotting using anti-mouse AA IgG antibody, was adjusted to 1.125 g/ml with KBr and ultracentrifuged for 21 hrs at 55,000 rpm in a 60Ti rotor at 10°C. SAA-HDL containing fractions were concentrated and dialyzed against PBS, pH 7.4.

2.19. Cell Culture

This was performed under strict aseptic conditions. Harvested peritoneal exudate containing monocytoid and polymorphonuclear leukocytes (3.5 X 10^6 cells/ml supplemented RPMI-1640) were incubated in Rose chambers at 37°C in the presence of 5% CO2. 2 hrs later, the chambers containing peritoneal cells were washed 3 times with RPMI-1640 to remove the non-adherent cells. Rose chambers were filled with fresh RPMI-1640 medium, supplemented with 300 mg/l L-glutamine, 10 mM HEPES buffer, penicillin (100 U/ml), streptomycin (100 mg/ml), 10% bovine calf serum, and SAA-HDL (3.5 and 7.0 mg) or 0.4 ml of normal mouse serum, and reincubated as mentioned before. After 4 day incubation, the supernatants were removed, and microcentrifuged for 10 min (14,000g). The sediments were spread on gelatin coated glass slides, air dried and stained with Congo-red as previously described. The cells attached to the glass cover slip in the Rose chambers were air-dried and stained with alkaline Congo-red, RAA and thioflavin S, as described before. Cytocentrifuged peritoneal cells were also stained with RABU and examined using indirect immunofluorescence technique as previously described.

In a parallel experiment, 0.3 and 0.9mg dosages of RABU IgG antibody were added to the chambers already supplemented with the medium and 7.0 mg of SAA-HDL before the 4 day incubation period, and the supernatants and cells were treated in a similar fashion. The peritoneal cells from normal mice used in a control group were incubated with 7.0 mg SAA-HDL, supplemented in the medium, for 4 days at 37°C with 5% CO2, then processed in a similar fashion.

RESULTS

3.1. AEF Activity of Mouse Crude and Electro-Eluted Fractions

The crude murine AEF (0.1 mg/mouse) with or without 10 mM $CaCl_2$ showed AEF activity, although, addition of $CaCl_2$ increased significantly the potency of AEF (Table 3.1, groups 1,2). Without the $AgNO_3$ stimulus, this dosage of AEF proved to be non-amyloidogenic (Table 3.1, group 3).

Upon primary purification of crude AEF, following acidification and gel filtration, four fractions F1 to F4 were obtained (Fig. 3.1). The fractions were concentrated and assayed for AEF activity (0.2 mg protein/mouse). F1 showed potent AEF activity (80 to 100% of the splenic follicles had 2+ to 3+ AA), marginal AEF activity was present in F2 (1 to 2% of the spleen follicles had ± to 1+ AA) and none in F3 and F4 fractions. Addition of 10mM $CaCl_2$ and 10 mM dithiothreitol (DTT) to F2 to F4 enhanced significantly the AEF potency of F2 only (50 to 60% of the spleen follicles had 1+ to 2+ AA); the other two fractions remained negative for AEF activity.

In SDS-PAGE, F1 demonstrated at least 18 peptides of molecular weights greater than 21.5K (protein standard, soybean trypsin inhibitor) and one minor band of 5.5K (Fig. 1, inset). F2 (Fig. 3.1, inset), which was also positive for AEF, contained 5 prominent peptides of molecular weights under 31.5 K (protein standard, bovine carbonic anhydrase). We chose to electro-elute the peptides from F2 and test their AEF activity. Since boiling almost completely abolishes AEF activity (Axelrad and Kisilevsky 1980; also unpublished), the F2 fraction was incubated for 2 hr at 37°C with 2x Laemmli's buffer (see material and methods) and electrophoresed on a 15% SDS-PAGE gel. The Coomassie stained peptides, as indicated in Fig. 3.1 (inset, EP-AEF-a to EP-AEF-c) were electro-eluted and bioassayed in approximately 100 μg dosage per mouse with added $CaCl_2$ and DTT along with 4 s.c. injections with 0.5 ml of 1% $AgNO_3$. Only EP-AEF-a

demonstrated AEF activity; mice tested with EP-AEF-a but without the $AgNO_3$ stimuli failed to demonstrate splenic AA deposits (Table 3.1, groups 4-7). Similarly as shown in Table 3.2, murine ubiquitin by itself was not amyloidogenic (group 1). Moreover, neither 4 s.c injections with 1 or 2% $AgNO_3$ alone (Table 3.2; groups 2,3) nor i.p or i.v. injections with azocasein, human hemoglobin or bovine serum albumin in conjunction with 4 s.c injections with 1% $AgNO_3$ (groups 4-6), induced splenic AA amyloid in mice. Thus the AEF activity, detected in EP-AEF-a, was specific.

The reason for using $CaCl_2$ and and dithiotritol (DTT) as additives to the AEF inocula stems from our previous work (unpublished). We found that AEF activity in Alzheimer brain extract, kept at 4°C, with these two additives, remained essentially unaltered for up to 3 months. Subsequent to these initial studies with the semipurified AEF (Table 3.1, groups 4-7), DTT has now been deleted from the test AEF inocula. DTT alone does not appear to enhance the AEF activity, whereas addition of $CaCl_2$ does.

3.2. Amino Acid Sequencing and Western Immunoblotting

EP-AEF-a, which on SDS-PAGE appeared to be a homogeneous peptide, was subjected to 42 cycles of NH_2-terminal amino acid sequencing and the resulting sequence was found to be identical to that of mammalian ubiquitin (Fig. 3.1B) (Wilkinson 1988). 48 cycles of amino acid sequencing of AD brain-derived ubiquitin also confirmed its identity (Fig. 3.5) (Alizadeh-Khiavi et al. 1990a). In SDS-PAGE, EP-AEF-a co-migrated with bovine ubiquitin, and both the murine and bovine ubiquitin (BU) samples and in Western immunoblottin, immunoreacted with RABU (Fig. 3.1B). No detectable staining was observed when these two electro-transferred peptides, as shown Alzheimer brain derived ubiquitin (Alizadeh-Khiavi et al. 1990a), were reacted with RABU absorbed with BU. Thus the immunoreactivity of RABU with the ubiquitin samples was specific.

3.3. Large Scale Purification of Murine Tissue Derived Ubiquitin and its AEF Activity

Since heat-treatment affects significantly AEF activity (Axelrad and Kisilevsky 1980), the ethanol-chloroform method (Matsumoto et al. 1984) was used for the purification of ubiquitin from the crude AEF (see materials and methods). The estimated yield of monomeric ubiquitin was less than 0.1 percent of the starting crude AEF. The CM-Sepharose eluted fraction migrated as a homogenous 5.5K peptide on a 15% SDS-PAGE gel (Fig. 3.2; lane 6), immunoreacted with RABU (Fig. 3.2, lane 5), similar to that of EP-AEF-a (Fig. 3.1B) and showed AEF activity in a concentration dependent fashion in the mouse bioassay (Fig. 3.3). Mice received either one s.c. injection of 2% $AgNO_3$ or 4 s.c injections of 1% $AgNO_3$ along with various i.p. doses (3,10,50 and 100 μg/mouse) of the tissue derived monomeric ubiquitin. Both the amounts of AA (± to 3+) and the percentage of AA positive follicles corresponded with the increasing ubiquitin doses (Fig. 3.3). At lower ubiquitin doses (3 and 10 μg) a relatively higher percentage of mice receiving one injection of 2% $AgNO_3$ showed splenic AA deposits than those stimulated with 4 injections of 1% $AgNO_3$. Although the AA grading appeared comparable in both cases, mice stimulated with multiple s.c. injection of 1% $AgNO_3$ showed a relatively higher percentage of AA positive splenic follicles than those receiving 2% $AgNO_3$. Regardless of these variations 4 $AgNO_3$ injections (1 or 2%) alone, as also reported previously (Ali-Khan et al. 1988b, Alizadeh-Khiavi et al. 1990a), did not induce splenic AA deposition (Table 2.2).

Commercially available BU purified by the heat-treatment method (Ref. Sigma Catalogue, 1990, pp. 1028), was assayed for AEF activity in 0.1, 1.0 and 10.0 mg/mouse dosages in conjunction with 4 s.c. injections of 1% $AgNO_3$. In PBS, none of these ubiquitin dosages showed AEF activity. With 10mM $CaCl_2$, 1 of 3 mice with 0.1 mg, 7 of 11 mice with 1.0 mg and 2 of 3 mice with 10 mg BU dosages generally demonstrated residual ± to 1+ grades of splenic AA.

Calf thymus derived ubiquitin (lot No. 701799), also purified by the heat-treatment method (Calbiochem, CA, U.S.A. Catalog 1990-1991, pp 225), failed to demonstrate AEF activity. Heat-treatment drastically deteriorates the AEF activity in both ubiquitin and as shown previously with the crude AEF preparations (Axelrad and Kisilevsky 1980). Intrinsic proteolytic activity of ubiquitin is also abolished by heat treatment (Murti et al. 1988).

3.4. Purification of Murine Ubiquitin Adducts and Their AEF Activity

The crude mouse AEF was immunoblotted using RABU or a reference anti-ubiquitin antibody (Fig. 3.2). Both these antibodies showed identical immunoblot patterns, that is a 5.5K monomeric ubiquitin band and at least 5 major and several minor large molecular weight proteins (>40K) reacting with RABU (Lane 1). The large molecular weight RABU positive proteins may represent ubiquitin adducts. Specificity of the immunostaining was determined with RABU absorbed with BU. As demonstrated previously (Alizadeh-Khiavi et al. 1990a & 1990b, Chronopolous et al. 1990b; also see below), the absorbed antibody failed to bind to the electro-blotted crude AEF or monomeric ubiquitin.

To ascertain whether ubiquitin adducts possess AEF activity both the adducts and monomeric ubiquitin were isolated from crude AEF using the immunoaffinity and exclusion chromatography methods (see materials and methods). On immunoblotting, both the resulting flow through (Fig. 3.2, lane 3) and the eluted (Fig. 3.2, lane 4) fractions contained a few similar ubiquitin adducts, and monomeric ubiquitin was present only in the eluted fraction indicating a stronger affinity between RABU and monomeric ubiquitin. Excessive amounts of ubiquitin adducts present in the crude AEF may also explain the persistence of ubiquitin adducts in the flow through fraction. In contrast, the 1M ethanolamine blocked Sepharose gel lacking the ligand, failed to bind to proteins present in the crude AEF (Fig, 3.2, lane 2). This suggests that the binding of monomeric ubiquitin and ubiquitin adducts to Sepharose gel conjugated to RABU was selective and specific, although this procedure under the present experimental conditions did not

completely deplete the ubiquitinated proteins from the crude AEF (Fig. 3.2, lane 3). The affinity purified fraction was further separated into two fractions P1 (void volume) and P2 (retarded fraction) by exclusion chromatography using Sephacryl S-200 gel and immunoblotted (Fig. 3.4). P1 contained at least two major and two minor large molecular weight adducts, while P2 contained mainly the monomeric ubiquitin (Fig. 3.4). Both these fractions demonstrated AEF activity in a dose-dependent manner (Table 3.3).

3.5. Purification of Alzheimer's Disease Brain Derived Ubiquitin and its AEF Activity

We have previously described the presence of AEF in crude brain extracts from AD patients (Ali-Khan et al. 1988b). In the present study, AD-Ub, was purified to apparent homogeneity (Fig. 3.5). Purification was achieved by sequential chromatography of the crude brain extract (lane 2), on DEAE-Sepharose (lane 3), Sephacryl S-200 HR (lane 4), and CM-Sepharose (lane 5). On a 15% SDS-polyacrylamide gel, the CM-Sepharose eluted purified AD-brain derived peptide had an apparent mol. wt of ~5.5 kDa. At most this peptide is present in trace amounts (approximately less than 0.1% of the total starting protein) in the crude extract, which contained approximately 37 bands ranging in mol. wt. between ~5.5 to >97.4 kDa.

On a 15% SDS-polyacrylamide gel, the purified ~ 5.5 kDa peptide showed an identical electrophoretic mobility as that of bovine ubiquitin (BU, lane 6). In Western immunoblotting both the ~ 5.5 kDa peptide (lane 7), and BU (lane 8), reacted with RABU. Immunostaining of these two peptides was abolished when RABU was absorbed with BU (Alizadeh-Khiavi et al. 1990a & 1990b, Chronopoulos et al. 1990a & 1990b). Furthermore, partial amino acid sequence analysis of the AEF positive ~ 5.5 kDa peptide derived from AD brain and murine tissue indicated a striking homology with eukaryotic ubiquitin (Fig. 3.5; Alizadeh-Khiavi and Ali-Khan 1990, Alizadeh-Khiavi et al. 1990a & 1990b, Rechsteiner 1987).

The mouse bioassay was used to determine AEF activity in the crude brain extract, and the two ~ 5.5 kDa peptide (heat-treated or ethanol-chloroform treated) preparations (Table 3.5). The crude extract showed potent AEF activity (group 1). Since addition of 10 mM $CaCl_2$ enhanced the AEF activity of purified murine and bovine ubiquitin, we next examined concentration-dependent amyloidogenic potency of the ~ 5.5kD peptide isolates with or without $CaCl_2$. Ethanol-chloroform treated ~ 5.5kDa peptide with added $CaCl_2$, at both 0.01 mg and 0.02 mg/mouse dosages, induced heavy amyloid deposition (group 2). In sharp contrast, a 75% reduction was observed in the AEF potency when $CaCl_2$ was omitted from the highly amyloidogenic dosage (0.02 mg/mouse, group 2). Interestingly, Fried et al., showed a similar effect of $CaCl_2$ on the in vitro proteolytic activity of ubiquitin (Fried et al. 1987). It was suggested that ubiquitin might function as a free protease and its activity might be regulated by $CaCl_2$.

Heat-treated ~ 5.5 kDa peptide, even with added $CaCl_2$, showed much reduced AEF activity (Table 3.5, group 3). When $CaCl_2$ was omitted from the heat-treated ~ 5.5kDa peptide (0.2 mg/mouse) it failed to show AEF activity (group 3). Similar results were obtained with crude murine extracts exposed to heating (Axelrad and Kisilevsky 1980), and also with the two murine and bovine ubiquitin preparations which were purified by exposing the crude extract to heat-treatment (Alizadeh-Khiavi and Ali-Khan 1990, Alizadeh-Khiavi et al. 1990a). In general, exposure of crude AEF to boiling abolishes most of the AEF activity (Axelrad and Kisilevsky 1980).

3.6. AEF Bioactivity in Recombinant Wild Type Ubiquitin and its Mutants

Recombinant WT UB and 5 of 6 mutagenized ubiquitin species, except one with a glycine to alanine substitution at position 76 (Table 3.6; group 6), induced accelerated splenic amyloid deposition in mice in 48 h (Table 3.6). Furthermore, AEF activity in mice was reduced in lysine-

48 mutant when tested along with 3 daily s.c. injections of 1% $AgNO_3$ solution, and was absent with a single s.c. injection of 2% $AgNO_3$ on a 48 hr induction basis (group 7). This substitution was not capable of inducing AA amyloid in mice after 48 hr. There were no amyloid deposits in the spleens of mice in control groups 8 through 11.

3.7. Effect of Anti-Ubiquitin Antibody on the Biological Activity of Crude Murine AEF

We also investigated whether RABU would block the AEF activity in the crude AEF. If so, this would indicate that ubiquitin is functionally analogous to AEF. Results in Fig. 3.8 show that RABU, whether passively transferred 24 hr prior to the challenge with crude AEF or injected after overnight incubation with crude AEF, completely abolished the biological activity of crude AEF in a dose-dependent manner. The optimal concentration of RABU required for complete abolition of AEF activity was approximately 2-fold higher in the antibody passive transfer experimental group, than in that of overnight incubation with RABU. Normal rabbit IgG, used as the control, did not affect AEF activity in the crude AEF. These results further confirm that AEF activity is associated with ubiquitin and its adducts.

3.8. Anti-Ubiquitin Antibody Neutralization of AEF Bioactivity in Recombinant Ubiquitin

Anti-ubiquitin antibody almost completely abolished the AEF activity of Rc-WT UB (Fig. 3.9; group 1). Normal rabbit IgG at a matched dose did not have any neutralizing effect on Rc-WT UB (group 2) and the amyloidogenic response in this group was similar to the control group which received only Rc-WT UB with no antibody treatment (group 3).

3.9. Pattern of Ubiquitin Response in Activated Peritoneal Cells

To investigate the pattern of ubiquitin response, cytocentrifuged peritoneal cells from protease-peptone and AHC-infected mice were reacted with RABU and analyzed by IFA. Almost all the mature (Fig. 3.10a-c) and immature (Fig. 3.10d) polymorphonuclear leukocytes (PMN) between day 1 to 4 weeks postinfection, contained intensely fluorescent RABU-positive granules; in some PMNs the entire cytoplasm appeared as a homogeneous RABU-positive fluorescent mass (Fig. 3.10b). The percentage of ubiquitin positive macrophages containing discrete immunopositive granules increased from 38% at day 1 to 54% at 4 weeks (Fig. 3.10c). Clusters of extracellular fluorescent granules were also seen among the cytocentrifuged leukocytes. These granules were apparently released from the leukocytes during cytocentrifugation. Peritoneal cells, obtained 72 hr after an ip injection with protease-peptone, were used as controls. A majority (87%) of the macrophages contained only nuclear immunopositive granules while the remaining cells contained both nuclear and cytoplasmic granules (Fig. 3.10a). PMNs were rare cellular infiltrates in the control preparations and most of them showed dull and diffuse cytoplasmic fluorescence unlike the PMN from the AHC-infected mice (Fig. 3.10a). The immunostaining for ubiquitin was specific; neither the preimmune rabbit serum nor the RABU absorbed with BU immunoreacted with the activated leukocytes (Alizadeh-Khiavi et al. 1990 b).

Ubiquitin pattern in amyloidotic mouse peritoneal cells was also studied by immunoelectron microscopy. Immature (Fig. 3.11a) as well mature macrophages (Fig.3.11b) contained focal and diffuse intracytoplasmic ubiquitin, although the former cells demonstrated a more prominent presence of ubiquitin on some electron-dense compartments comparable to lysosomes in their size. Also, some released gold labelled electron dense vesicles were present in the vicinity of some

macrophages (Fig. 3.11a; arrowhead). Electron-transparent and electron-dense compartments (some with a double tracked membrane) inside peritoneal neutrophils were also intensely decorated with RABU (1:10 and 1:20 dilutions; 0.3 mg/ml) (Fig. 3.11c,d). RABU preabsorbed with bovine ubiquitin did not bind to these cells (Fig. 3.11e,f). Moreover, peritoneal cells from normal mice did not show any significant binding to RABU (Fig. 3.16b,c).

3.10. Colocalization of AA Amyloid and Ubiquitin in Mouse Amyloidotic Spleens

To examine the spatial relationship between ubiquitin and AA in AD-UB induced amyloidosis, mouse spleen sections were first stained with RABU and then counterstained with thioflavin S to detect amyloid deposition. Light to dark brown RABU-positive deposits were found both intracellularly but mainly interstitially in both the AA-positive and AA-negative PFA (Fig. 3.7a-c). No staining was observed when the AA positive spleen sections were treated with either the preimmune rabbit serum or RABU absorbed with ubiquitin (Fig. 3.7d). Thus ubiquitin is found at sites of AA deposition. Also noticeable was the gradient in the intensity of RABU-positive brown staining in the spleen parenchyma. It was relatively high in the PFA containing AA (as opposed to normal PFA, Fig. 3.7e) and decreased gradually towards the red pulp (Fig. 3.7a,c). In contrast, spleen sections from normal mice showed focal clusters of RABU-positive cells, adjacent to the trabeculae (Fig. 3.7e) and no brown deposits were present in the PFA. This suggests that local ubiquitin concentrations may play a role in amyloid deposition.

Ultrathin sections from 7 day post-infected mouse spleens were also stained with RABU and RAA antibodies and examined by an electron microscope. Double labelling of sections was not possible, since both primary antibodies, RAA and RABU, were raised in rabbits. Instead serial

sections from the same block were stained with RAA and RABU separately and their binding patterns were compared. Etching ultrathin sections with hydrogen peroxide retrieved the immunoreactivity with both antibodies. Overnight incubation with RABU as the primary antibody was essential for better antigen-antibody binding (1:20 dilution, 0.3 mg/ml). With RAA (1:400 and 1:800; 10 mg/ml) 2 hr incubation produced satisfactory results.

Amyloid deposits were predominantly large and were located extracellularly usually adjacent or attached to the surface of reticuloendothelial cells, neutrophils, and macrophages. A few small intracellular amyloid masses were also present in some macrophages. While RAA decorated amyloid fibrils only (Fig.3.12a), an indication of AA type amyloid, RABU bound to both amyloid fibrils as well as other cellular structures such as nuclear chromatin (Fig. 3.13-15). Both RAA and RABU bound to the extracellular as well as intracellular amyloid masses. Moreover, in some cases (Fig. 3.15a) RABU decorated amorphous nonfilamentous structures as well as the amyloid fibrils. Based on noncovalent association of ubiquitin with murine AA type amyloid (Chronopolous et al. 1990a and 1990b), we suggest that these electron dense structures are primordial masses of aggregated SAA bound to ubiquitin, although more experimental evidence is required to support this hypothesis.

Spleen ultrathin sections treated with either RABU or RAA, preabsorbed with their respective antigens, did not contain any significant binding of gold particles (Fig. 3.12b, 3.15b). Furthermore spleen sections from normal mice showed no focal immunostaining in PFAs (Fig.3.16a).

3.11. SAA Response

Since neither crude AEF nor ubiquitin alone, when administered in amyloidogenic dosages, induced amyloidogenesis in mice (Table 3.1, group 3; Table 3.2, group 1; Table 3.6, group 8) we investigated their potential in the induction of SAA, the precursor protein of AA amyloid. Neither crude AEF, nor ubiquitin induced any significant serum SAA elevations at 16 hr (Table 3.4; groups 3,4; Table 3.7, group 1). In contrast, SAA elevation was higher than 500-fold above the basal level, in the LPS or $AgNO_3$ stimulated mice (Table 3.4, groups 1,2; Table 3.7, groups 8,9); while PBS alone did not cause any SAA synthesis in mice (Table 3.4, group 5; Table 3.7, group 10). On the other hand, none of the recombinant mutagenized ubiqitin proteins were capable of inducing SAA response in C57 mice (Table 3.7; groups 2-7).

3.12. Role of Ubiquitin in In-Vitro Amyloidogenesis

Cytocentrifuged peritoneal cells from 3 weeks post-infected C57BL/6 mice stained with Giemsa contained a large population (greater than 79 percent) of monocytoid cells, while very few granulocytes were present. As previously indicated in materials and methods, nuclear morphology was used in leukocyte differentiation. Similar cells were rich in cytoplasmic ubiquitin as judged by indirect immunofluorescence using RABU: macrophages generally showed diffused intracytoplasmic fluorescence while all the PMNs contained distinct fluorescent granules.

Ubiquitin-rich peritoneal cells from amyloidotic 3 week AHC-postinfected mice were capable of generating amyloid deposits 4 days after culture in SAA rich medium (Fig. 3.18-20). These deposits were generally seen in the vicinity of macrophages and were absent from the supernatant fraction. 1 out of 13 and 1 out of 8 microscopic fields in X400 magnification

contained congophilic amyloid deposits from low (3.5mg) and high (7.0 mg) SAA-HDL containing chambers respectively. Immunostaining with RAA proved these to be AA type amyloid deposits (Fig. 3.20b); SAA-HDL also appeared to bind the cell surfaces (Fig. 3.20b). Thioflavin S staining was also positive, an indication of amyloid deposits (Fig. 3.20a). However, similar cells incubated in the same condition with added RABU antibody failed to generate any congophilic or thioflavin-positive deposits. These cells did not produce any amyloid when normal mouse serum was substituted for the SAA-HDL rich serum. Similarly normal mouse peritoneal cells were not capable of generating amyloid when they were incubated with SAA-HDL for 4 days.

FIGURES

Figure 3.1. (1A). Sephacryl S-200 HR chromatography of the pH5 supernatant obtained from amyloidotic murine liver and spleen extracts; the insets represent Coomassie-stained 15% SDS-polyacrylamide gel pattern of protein bands in the pooled F1 and F2 fractions (15 μg). **(1B).** Homogeneous 5.5 k AEF positive peptide (left) (5 μg) similar to the EP-AEF-a peptide in the F2 fraction, and Western blot analysis of the same peptide using anti-bovine ubiquitin IgG as the primary antibody; partial amino acid sequence of the 5.5 k AEF positive peptide is shown below.

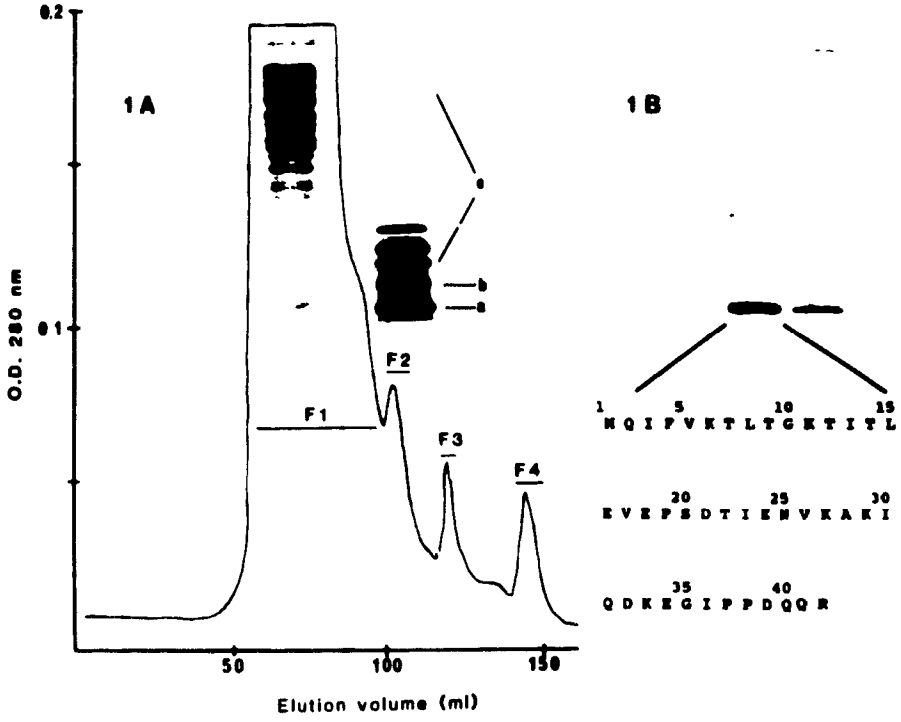

1A
1B
0.2
0.1
O.D. 280 nm
F1
F2
F3
F4
a
b
c
50
100
150
Elution volume (ml)
1 5 10 15
N Q I F V K T L T G K T I T L
20 25 30
E V E P S D T I E N V K A K I
35 40
Q D K E G I P P D Q Q R

Figure 3.2. Coomassie-stained 15% SDS-polyacrylamide gel profile of crude murine AEF (18 μg, lane 7), ubiquitin purified from this extract (5 μg, lane 6); and low (3.5 μg, lane 8) and high (7 μg, lane 9) molecular weight markers; lanes 1-5 represent the immunoblots using anti-bovine ubiquitin IgG antibody (RABU); lane 1, crude AEF; lane 2, eluate from the control 1M ethanolamine treated CNBr-activated sepharose gel incubated overnight with crude AEF; lane 3 (10 μg), flow through and 0.1M glycine-HCl, pH 2.8 containing 0.5M NaCl eluate (10 μg, lane 4) obtained from CNBr-activated sepharose gel conjugated to RABU and incubated with crude AEF, lane 5 (5 μg) murine ubiquitin; lane 8, low molecular weight markers (Combitek, Boehringer Mannheim Co.) from top to bottom: soybean trypsin inhibitor: 21.5 k, cytochrome C: 12.5 k, aprotinin: 6.5 k; lane 9, high molecular weight markers (Bio-Rad Co.) from top to bottom: phosphorylase B: 97.4 k, bovine serum albumin: 66.2 k, ovalbumin: 42.69 k, bovine carbonic anhydrase: 31 k, soybean trypsin inhibitor: 21.5 k, egg lysozyme: 14.4 k.

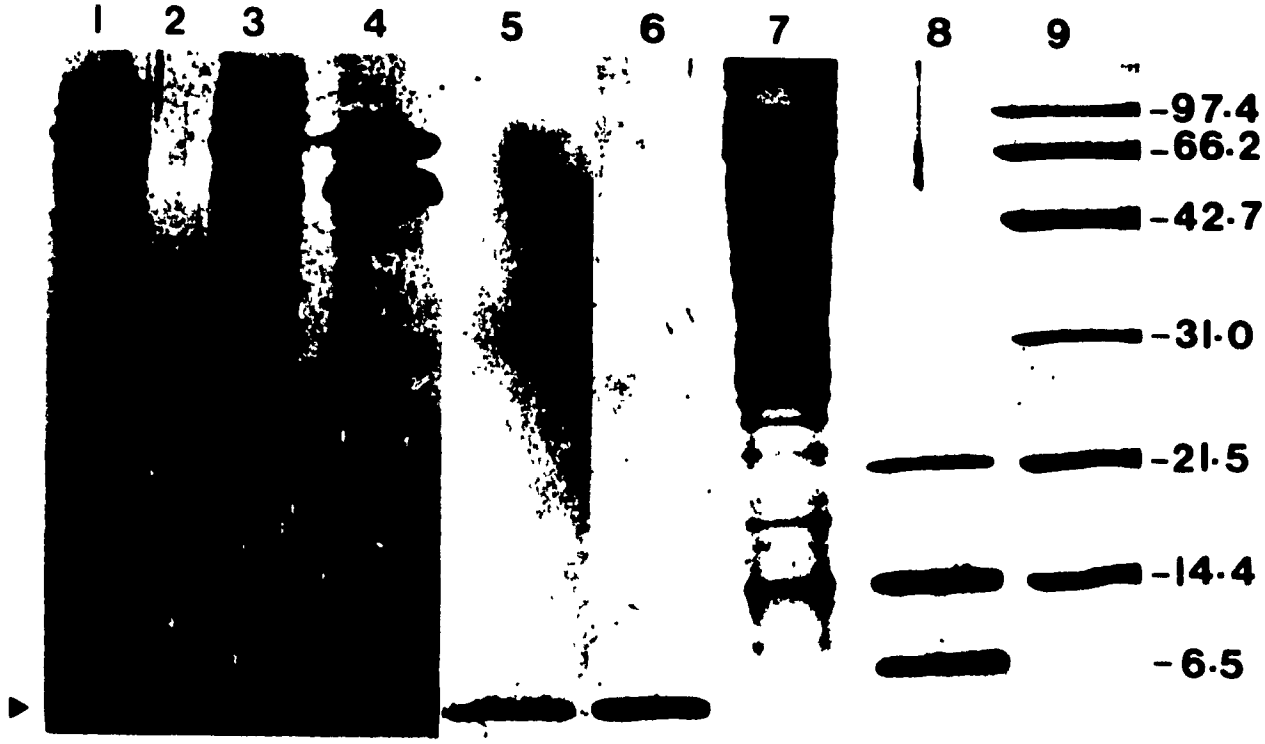

1
2
3
4
5
6
7
8
9
97·4
66·2
42·7
31·0
21·5
14·4
6·5

Figure 3.3. Shows dose-dependent AEF activity of murine tissue-derived ubiquitin in 100 mM phosphate buffer saline, pH 7.4 with 10 mM $CaCl_2$. Groups of mice received intraperitoneally 3,10,50 and 100 μg of ubiquitin in conjunction with either one subcutaneous (s.c) injection of 0.5 ml of 2% $AgNO_3$ (open circle) or four daily s.c injections of 0.5 ml of 1% $AgNO_3$ (closed circle). Mice in control group (filled square) received i.p 100 μg ubiquitin only, with no silver nitrate injections, and were sacrificed at 120 hr. Mice in the 2% and 1% $AgNO_3$ groups were sacrificed at 48 hr or at 24 hr after the last $AgNO_3$, respectively and their Congo-red-stained spleen sections graded for amyloid deposits (see materials and methods).

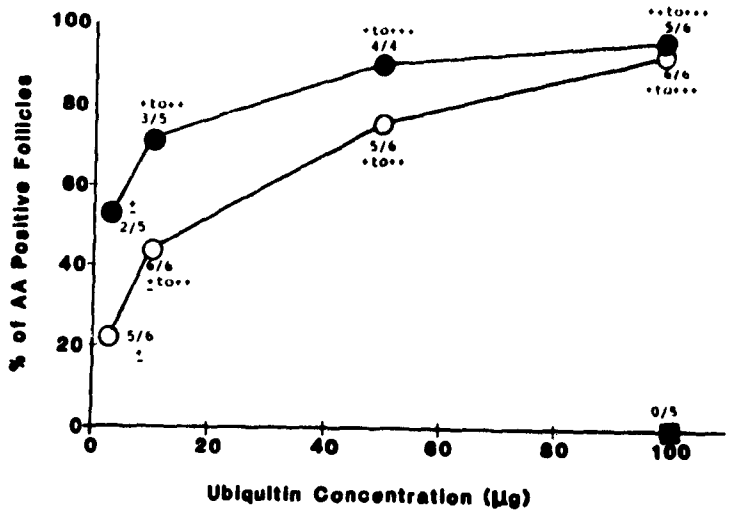

100
80
60
40
20
0
% of AA Positive Follicles
0
20
40
60
80
100
Ubiquitin Concentration (µg)
+to+++
4/4
++to+++
5/6
+to++
3/5
+
2/5
6/6
+to++
5/6
+to++
5/6
+
0/5

Figure 3.4. Sephacryl S-200 HR chromotography profile of 0.1 M glycine, pH 2.8 containing 0.5 M NaCl eluate obtained from CNBr Sepharose gel conjugated to rabbit anti-bovine ubiquitin IgG antibody (RABU) and incubated overnight with crude AEF. After thorough washing with 10 mM phosphate buffer pH 7.4 containing 0.5 M NaCl, glycine buffer was applied to elute the bound protein which was concentrated, fractionated into P1 and P2 and immunoblotted, using RABU. Two major and two minor large molecular weight ubiquitin adducts were seen in P1 and a single peptide, apparently monomeric ubiquitin of 5.5 k in P2.

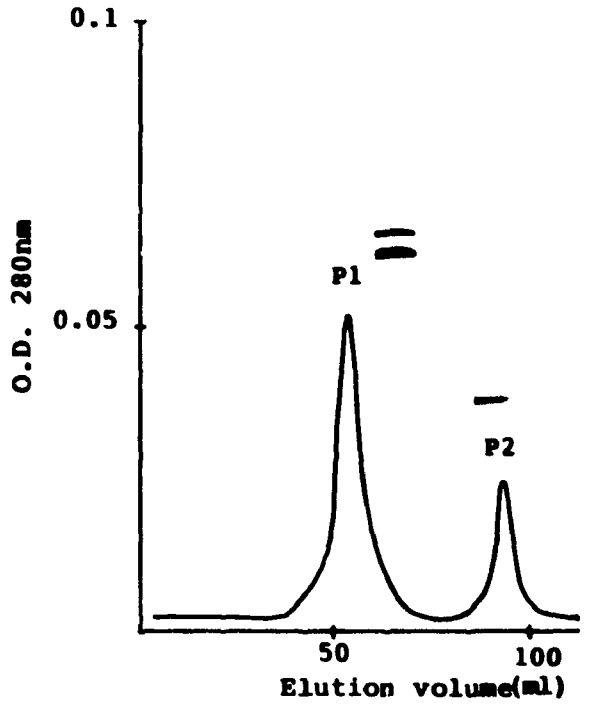

0.1
0.05
O.D. 280nm
P1
P2
50
100
Elution volume(ml)

Figure 3.5. Coomassie stained SDS-PAGE separated crude Alzheimer (AD) brain extract (lane 2, 15 μg) including its DEAE-Sepharose (lane 3, 15 μg), Sephacryl S-200 (lane 4, 10 μg), and CM-Sepharose eluted (lane 5, 6 μg) homogeneous amyloid enhancing factor (AEF). Electrophoretic mobility of AD-AEF (lane 5) is compared with bovine ubiquitin (lane 6, 5 μg). Western blots of AD-AEF (5 μg) and bovine ubiquitin (5 μg) are in lanes 7 and 8, respectively. Protein markers (lane 1, 10 μg), top to bottom phosphorylase B, 97.4 kD, bovine serum albumin, 66.2 kD, egg white ovalbumin, 42.6 kD, bovine carbonic anhydrase 31 kD, soybean trypsin inhibitor 21.5 kD and egg white lysozyme 14.4 kD (Bio-Rad, Toronto, Canada). Shown below is the partial amino acid sequence of the AD-AEF.

1 5 10 15 20
M Q I F V K T L T G K T I T L E V E P S

25 30 35 40
D T I E N V K A K I Q D K E G I P P D Q

45
Q R L I F A G K

Figure 3.6.a Congo red-stained, and **b** rabbit anti-mouse AA IgG antibody-immunostained amyloid deposits in splenic perifollicular areas (PFA) from mice exposed to 10 μg of purified AD-AEF. Note birefringent (arrow) and brown (arrowhead) amyloid deposits in the PFA; no immunostaining is present in the follicles (f) or the red pulp (r). X10

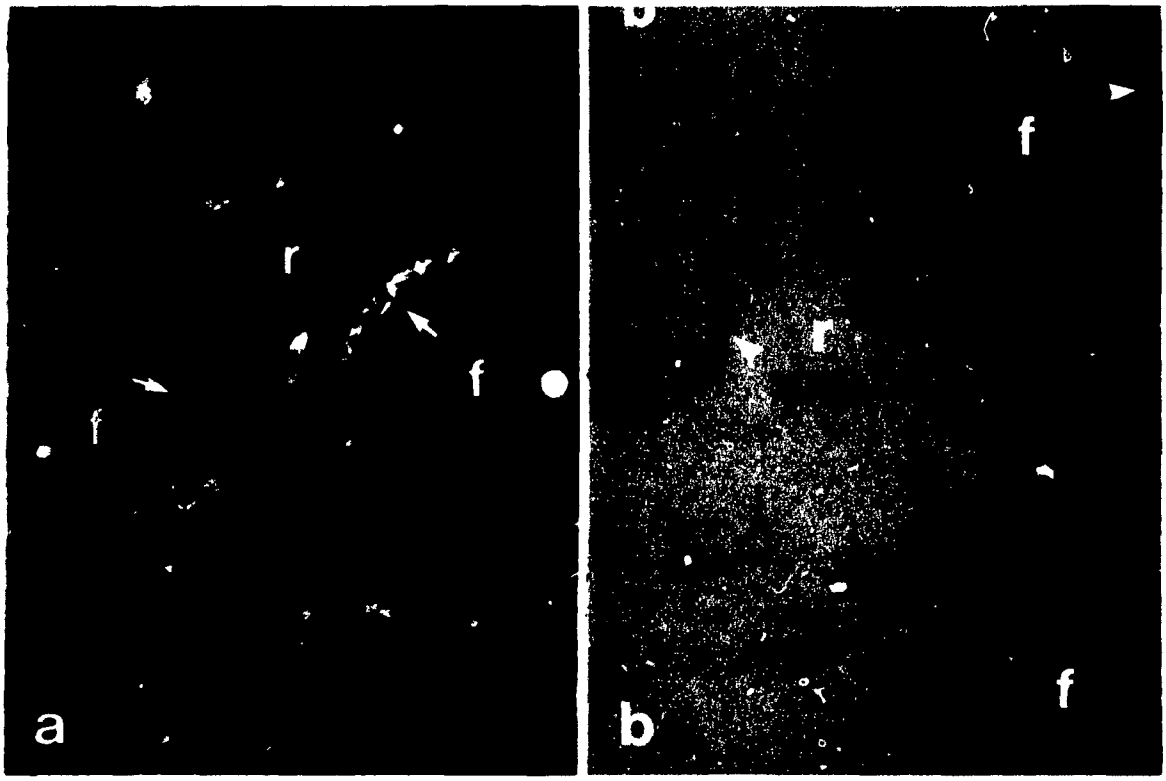

r
f
f
a
f
r
f
b

Figure 3.7. Spatial distribution of AEF and AA amyloid in splenic PFA from mice exposed to 10 μg of purified AEF. Note: **a** rabbit anti-bovine ubiquitin IgG antibody (RABU)-positive brown deposits (*arrow*) and **b** thioflavin S-positive fluorescent AA deposits (*arrowhead*) occupying similar PFA sites; no immunostaining is present in the follicle (*f*); **c** shows both the intracellular and the interstitial distribution of RABU-positive brown deposits in the PFA at a higher magnification; **d** shows complete abolition of immunostaining in a similar spleen section, as in **a-c**, after treatment with the RABU preabsobed with bovine ubiquitin; **e** a normal spleen section reacted with RABU, note focal brown deposits adjacent to the trabeculae (*small arrow*), and its absence from the PFA or the follicles (*f*). **a, b, d, e,** X16; **c** X40

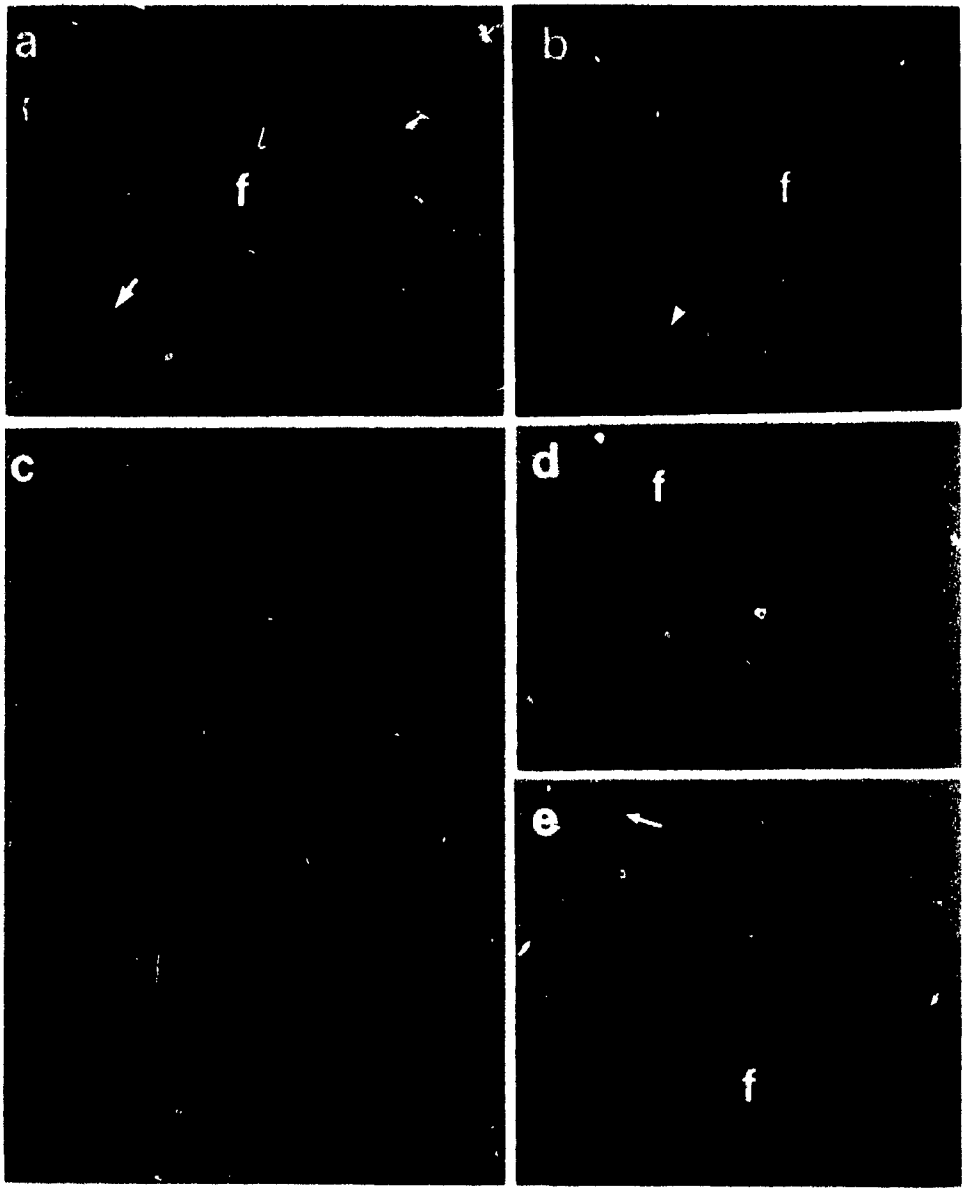

a
f
b
f
c
d
f
e
f

Figure 3.8. Effect of rabbit anti-bovine ubiquitin IgG antibody (RABU) on the amyloid enhancing factor (AEF) activity of crude AEF: groups of C57BL/6 mice received intraperitoneally (i.p) various amounts of RABU (closed circles) or 5 mg of normal rabbit IgG (filled square), 24 h before the i.p challenge with crude AEF (0.2 mg protein). In the second experiment, groups of mice received i.p crude AEF (0.2 mg protein) preincubated overnight with various amounts of RABU (open circle) or with 4 mg of normal rabbit IgG (open square). Mice in each group received one subcutaneous injection with 0.5 ml of 2% $AgNO_3$ and 48 h after their Congo red stained spleen sections were graded for AA deposits.

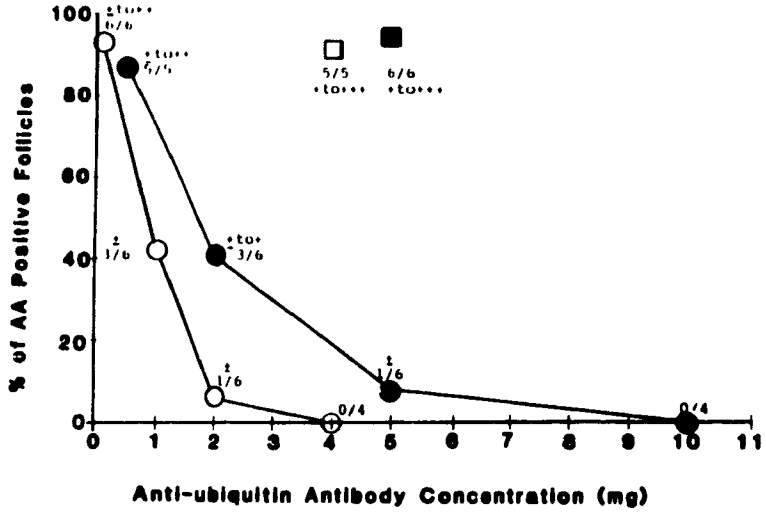

% of AA Positive Follicles
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
10
11
5/5
+to+++
6/6
+to+++
+to++
3/6
0/4
0/4
Anti-ubiquitin Antibody Concentration (mg)

Figure 3.9. Results of antiubiquitin antibody neutralization on the AEF activity of recombinant wild type ubiquitin in C57BL/6 mice; mice in groups 1 and 2 received intraperitoneally (ip) 100 μg Rc-WT UB incubated overnight with 4 mg of antiubiquitin IgG antibody or 4 mg, normal rabbit IgG, respectively. Mice in group 3 were injected ip with 100 μg Rc-WT UB. All mice received s.c 0.5 ml of 2% aqueous silver nitrate solution and 48 hr after they were sacrificed and their Congo red stained spleens were graded for AA deposition.

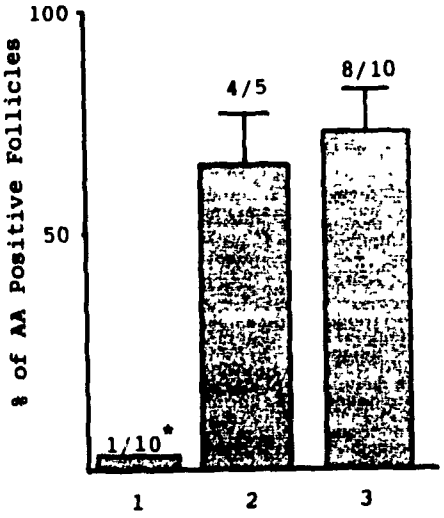

100
50
% of AA Positive Follicles
1/10*
4/5
8/10
1
2
3

Figure 3.10. Ethanol fixed cytocentrifuged peritoneal cells from proteose-peptone stimulated (1 ml of 10% solution,i.p) **a** or 250 alveolar hydatid cyst infected **b-d** mice, stained with rabbit anti-bovine ubiquitin IgG and fluoresceinated goat anti-rabbit Fab_2 antibody. Note the nuclear and cytoplasmic fluorescent granules (FG) in macrophages (*arrowheads*) and neutrophils (*arrows*) obtained 72 hr after proteose-peptone stimulation **a** or at 1 day **b** and 4 weeks **c,d** postinfection; also note an immature granulocyte **d** with a doughnut-shaped nucleus and peripherally cytoplasmic FG (x1000).

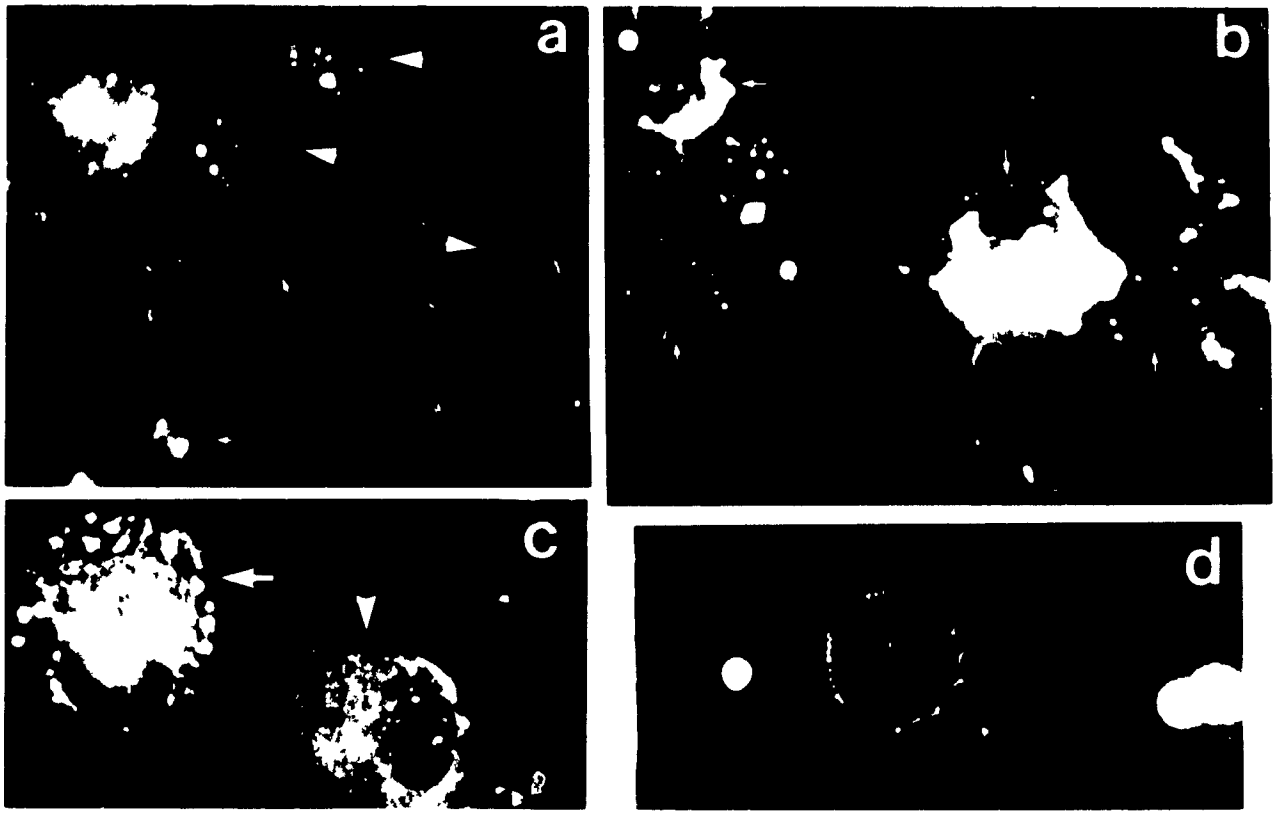

a
b
c
d

Figure 3.11. Immunolocalization of ubiquitin in peritoneal cells from C57BL/6 mice 7 day after infection with 250 alveolar hydatid cyst; the ultrathin section was treated with rabbit anti-bovine ubiquitin IgG (RABU) antibody. Note the nuclear and cytoplasmic gold particle labelling in both macrophages (**a, b**) and neutrophils (**c, d**); **a** an immature macrophage with focal gold labels on electron dense compartments and diffuse cytoplasmic as well as nuclear labelling. also note the extracellular gold labelled granule released from the same cell (*arrowhead*); **b** a mature macrophage labelled with 15 nm gold particles (*arrowhead*); **c** gold labelled electron transparent, and **d** electron dense intracellular compartments of polymorphonuclear leukocytes; **e, f** are neutrophil and macrophage cells respectively reacted with preabsorbed RABU antibody (see methods and material); bar represents 1 μm.

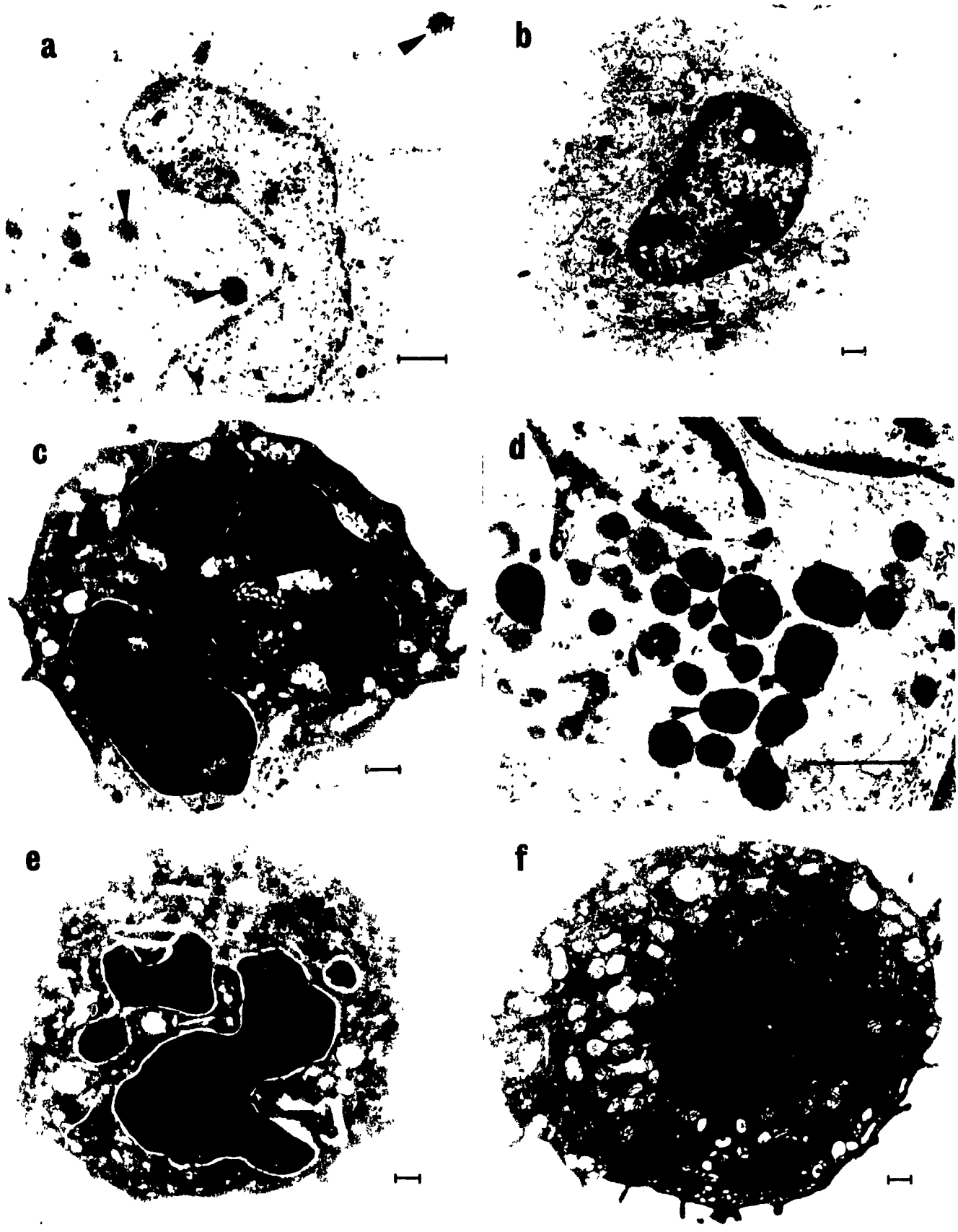

a
b
c
d
e
f

Figure 3.12.a AA type amyloid fibrils on the surface of a murine splenic reticuloendothelial cell detected by immunoelectron microscopy using rabbit anti-mouse AA IgG (RAA) antibody as primary antibody and biotinylated donkey anti-rabbit F(ab)2 as secondary antibody combined with strepavidin-auro probe (15 nm gold particles). The inset shows higher magnification of the amyloid fibrils and gold particles; **b** an amyloidotic murine spleen section containing AA type amyloid fibrils stained with preabsorbed RAA (see materials and methods section); inset shows a higher magnification of fibrils attached to a few gold particles; the bar represents 1 μm.

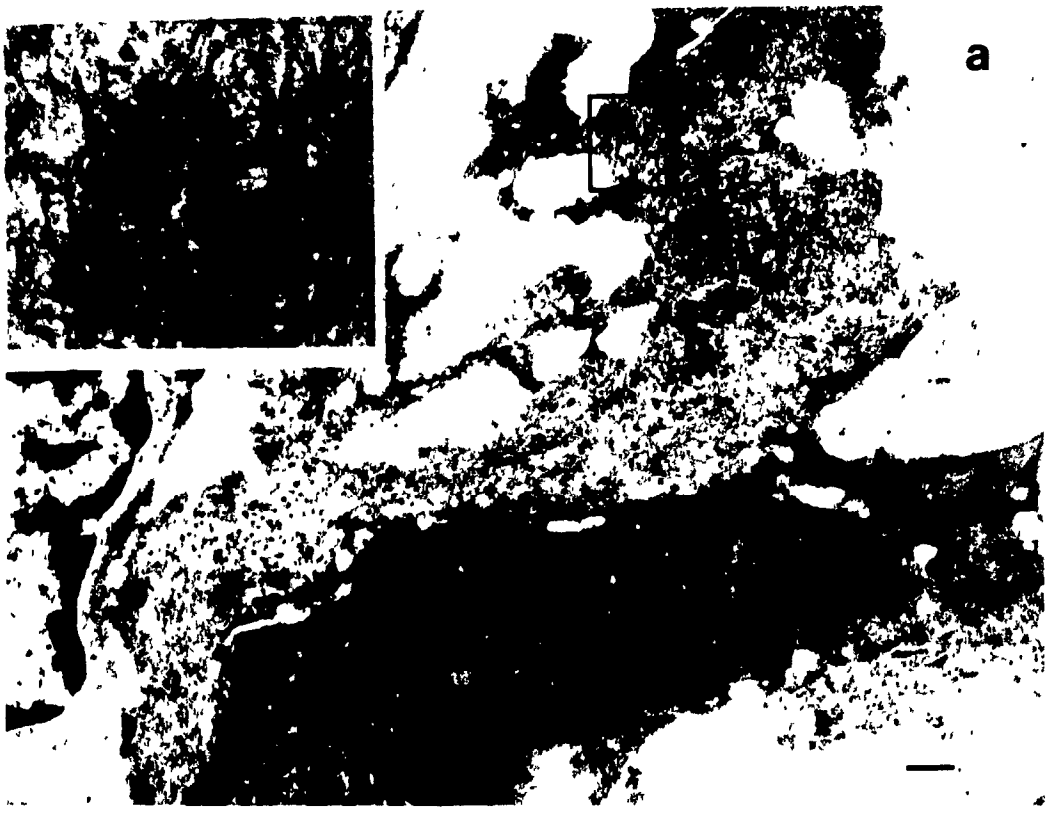
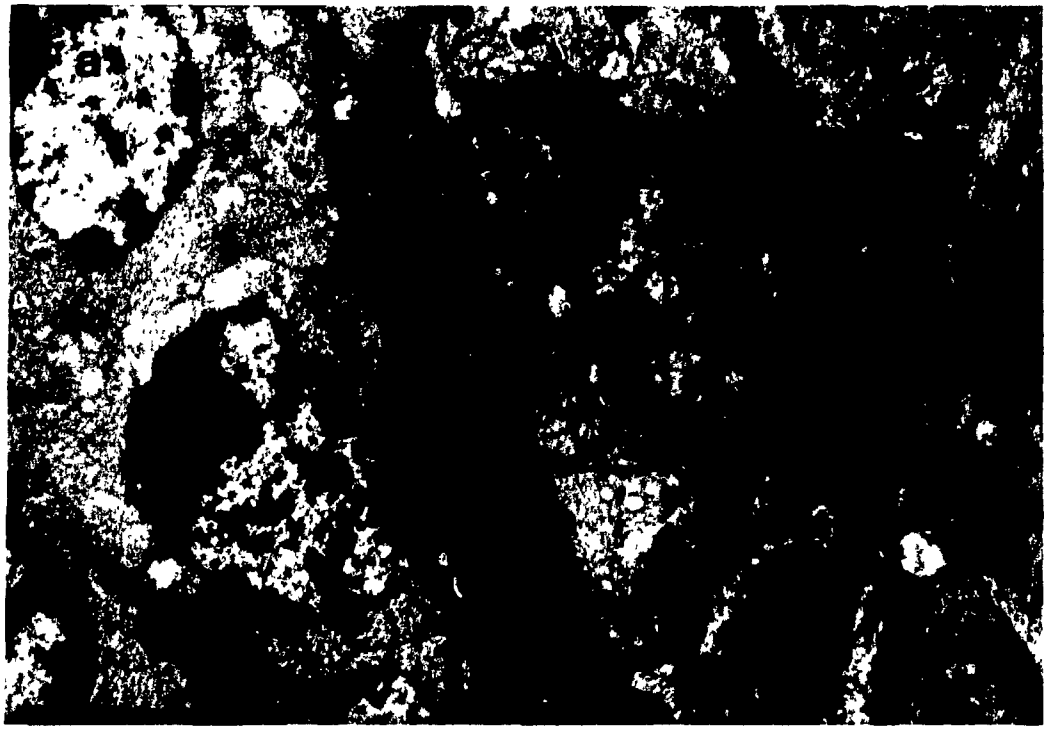

a

Figure 3.13. a Immunolocalization of ubiquitin bound to splenic AA amyloid in mice 7 days after infection with 250 alveolar hydatid cysts; The ultrathin section was treated with rabbit anti-bovine ubiquitin (RABU) IgG antibody. Notice the binding of 15 nm gold particles to amyloid fibrils deposited between two reticuloendothelial (RE) cells. Also note the binding of gold particles to the nuclear chromatin in these cells; **b** higher magnification of a part of the RE cell with adjacent extracellular amyloid fibrils bound to gold particles; inset shows a higher magnification of fibrils with attached gold particles. The bar in each picture represents 1 μm.


a

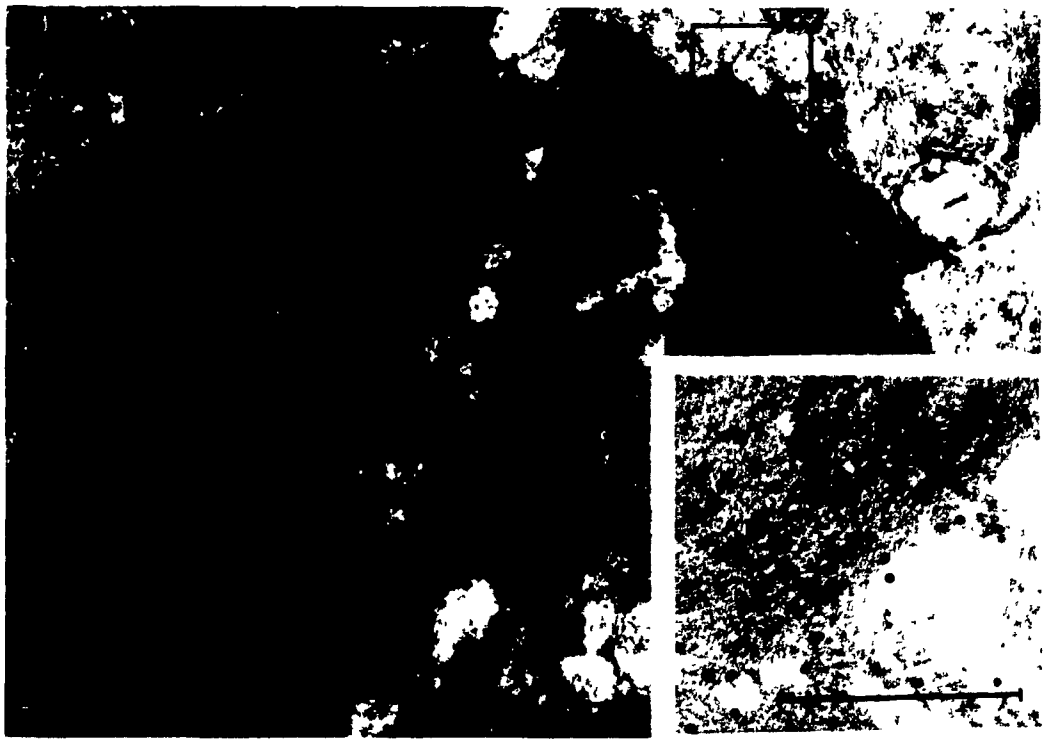


Figure 3.14. a Immunolocalization of ubiquitin bound to splenic AA amyloid from a mouse 7 days after infection with 250 alveolar hydatid cysts; the section was treated with rabbit anti-bovine ubiquitin (RABU) IgG antibody as the primary antibody and biotinylated donkey anti-rabbit F(ab)2 as the secondary antibody combined with strepavidin-auro probe. Note the binding of 15 nm gold particles to the amyloid fibrils deposited between two monocytoid cells. Also some gold is bound to the nuclear chromatin of cells; **b** binding of gold probe to amyloid fibrils deposited on the surface of a neutrophil (*arrowheads*), nuclear chromatin and to some extracellular structures; the bar represents 1 μm.

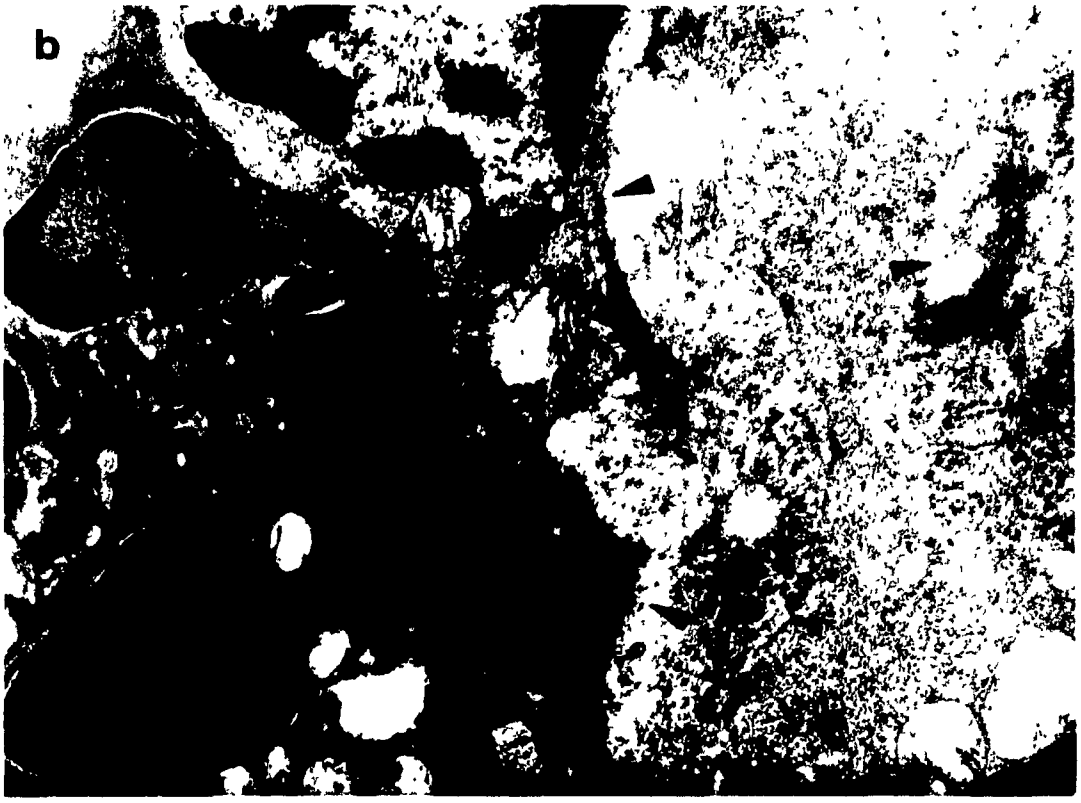

b

Figure 3.15 a Binding of ubiquitin to murine splenic amyloid fibrils (*large arrowhead*) and to amorphous intracellular electron dense vesicles (*small arrowhead*) demonstrated by immunoelectron microscopy using rabbit anti-bovine ubiquitin (RABU) IgG antibody. Also note that some of the immuno-positive compartments are surrounded by a double layered membrane; **b** amyloidotic spleen section reacted with the preabsorbed RABU (see materials and methods); note the presence of only a few gold particles bound to the amyloid deposit (*arrowhead*); the bar represents 1 μm.

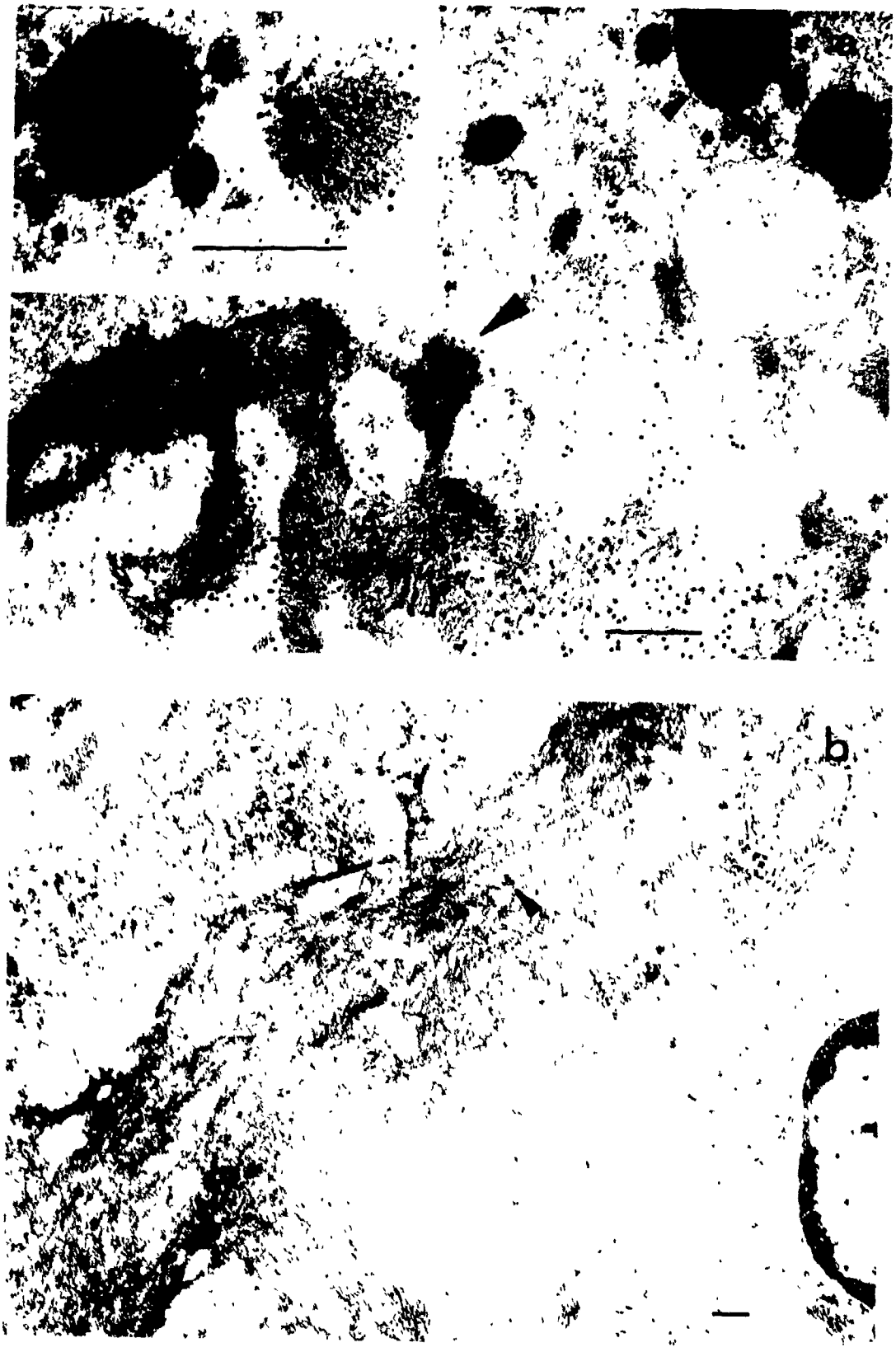
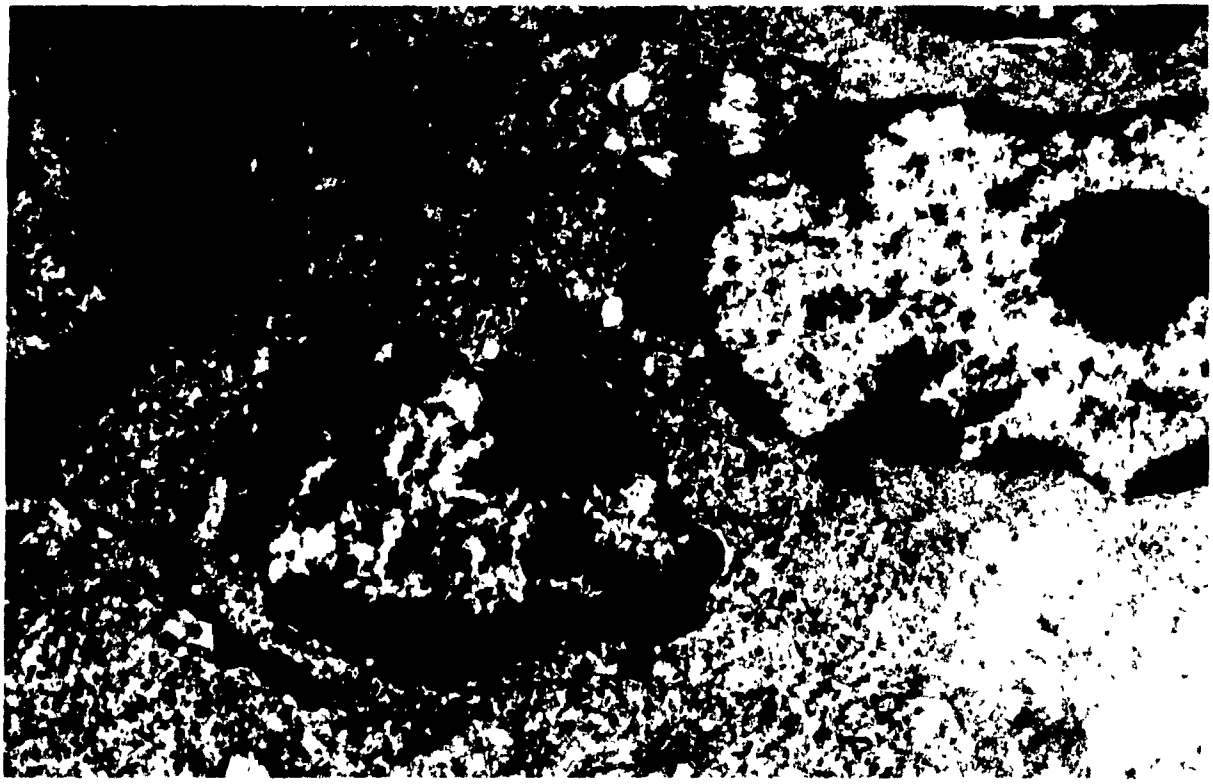
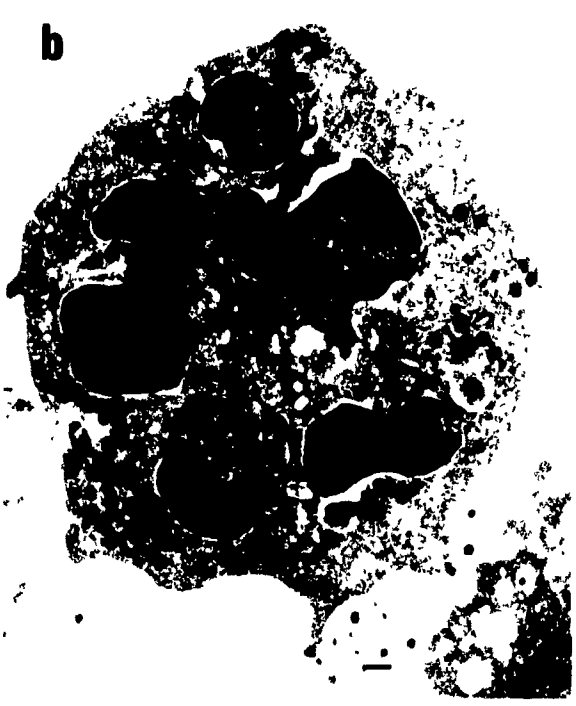

a
b

Figure 3.16. a Normal spleen section, **b** a peritoneal granulocyte, and **c** a monocytoid cell from normal C57BL/6 mice reacted with rabbit anti-bovine ubiquitin (RABU) IgG antibody, biotinylated donkey anti-rabbit F(ab)2, and auro-probe (strepavidin-gold) and examined by electron microscopy; notice the random distribution of cytosolic gold particles in spleenocytes, peritoneal granulocyte and monocytoid cell; the bar represents 1 μm.




b


c

Figure 3.17. Specificity of the immunoreactivity of the rabbit anti-AA (RAA) IgG and rabbit anti-bovine ubiquitin (RABU) IgG antibodies in binding to murine AA amyloid and ubiquitin as determined by western immunoblotting; lanes 1,3, and 5 contain mouse amyloid AA, while lanes 2, 4, and 6 contain ubiquitin bands; lanes 1,2 immunoreacted with RAA antibody, while lanes 3,4 were incubated with RABU as the primary antibody; lanes 5 and 6 were immunoreacted with absorbed RAA and RABU respectively; bars on the left are for standard markers from top to bottom: phosphorylase B: 97.4 k, bovine serum albumin: 66.2 k, ovalbumin: 42.69 k, bovine carbonic anhydrase: 31 k, soybean trypsin inhibitor: 21.5 k, egg lysozyme: 14.4 k; 5 to 10 μg protein was loaded per well.

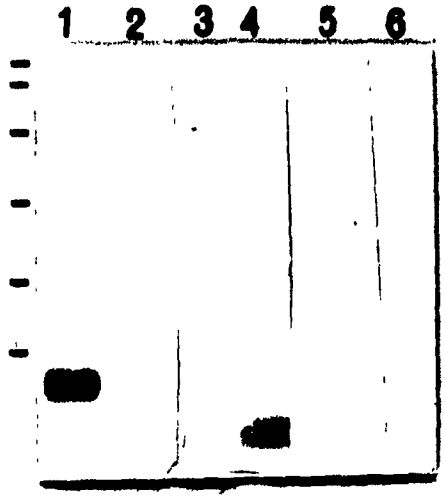

1
2
3
4
5
6

Figure 3.18. A large amyloid mass, approximately 250 μm in size, formed in vitro 4 days after culturing the harvested ubiquitin-rich peritoneal cells from 3 weeks post-infected C57BL/6 mice with SAA-HDL, stained with Congo-red; note its **a** congophilic appearance (*arrowhead*) under light microscopy; and **b** birefringent apple green appearance of the same field with polarized light microscopy (*arrowhead*); magnification X400.


a

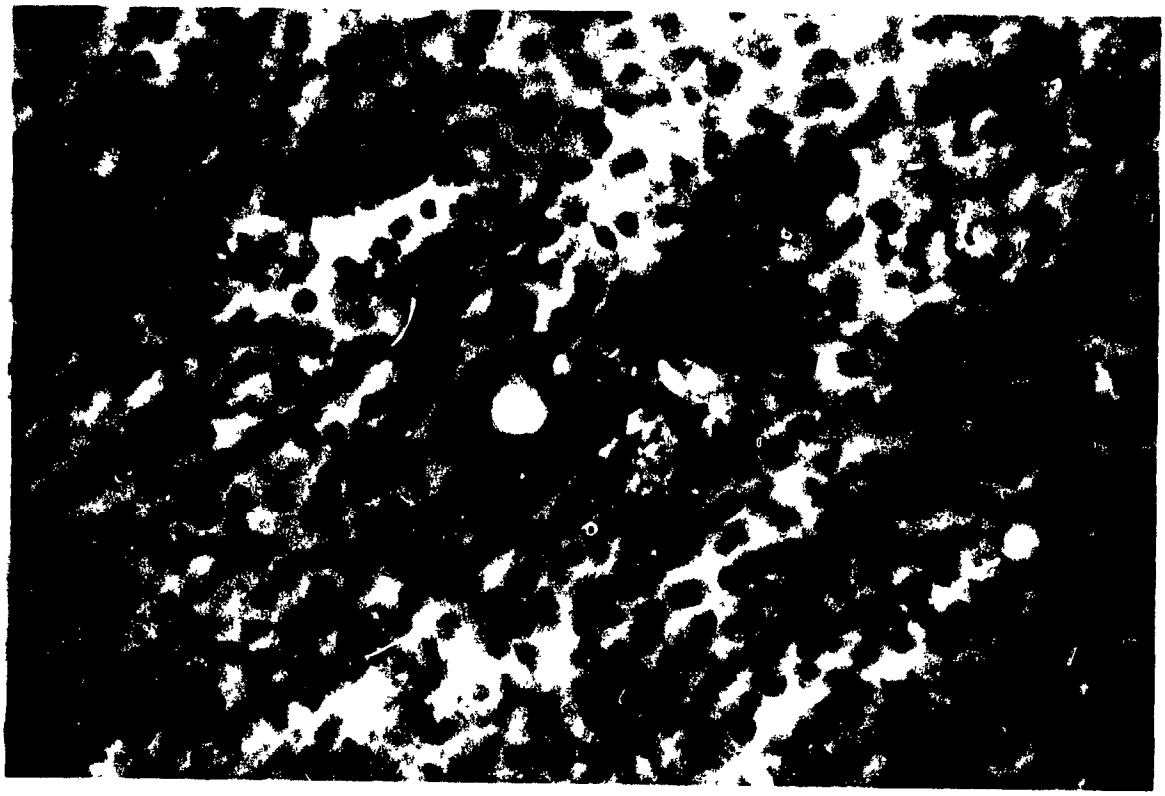
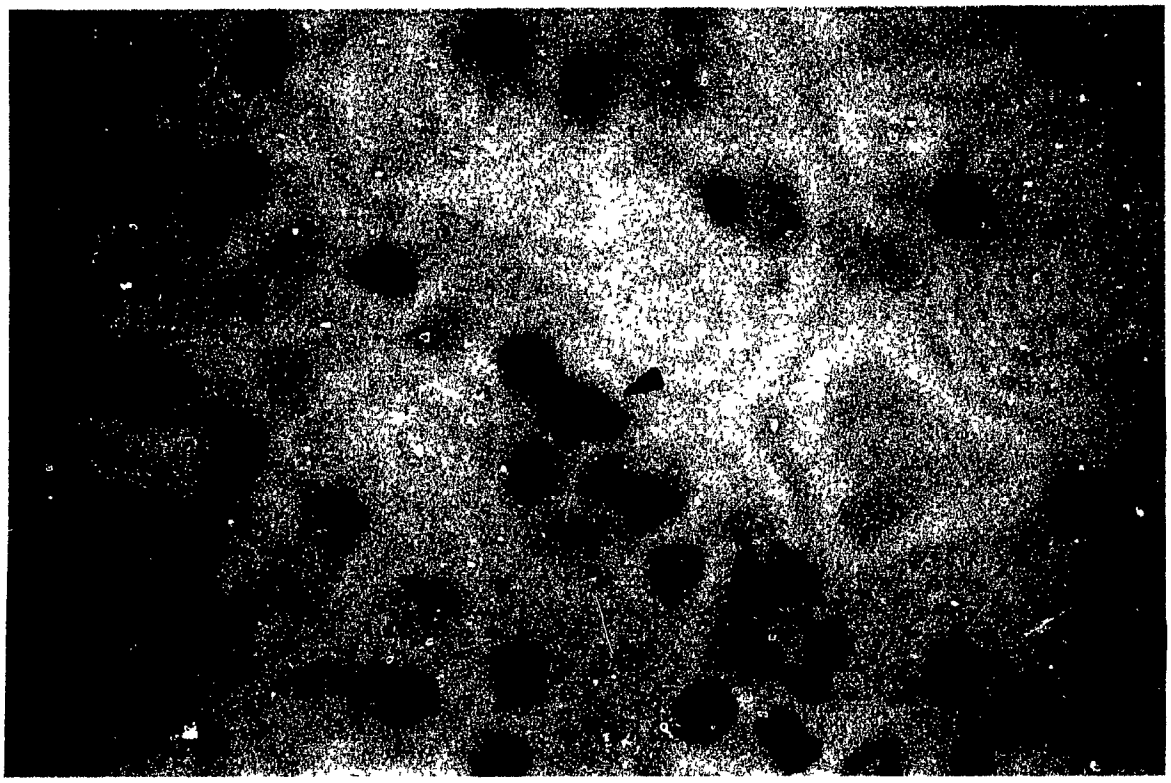


Fig. 3.19.a A small amyloid deposit, approximately 20 μm, observed among cultured ubiquitin-rich peritoneal macrophages from 3 weeks post-infected mice with 250 AHC; note its congophilic appearance under normal light (*arrowhead*), or **b** its green birefrengence with polarized light microscopy (*arrowhead*); magnification of X1000



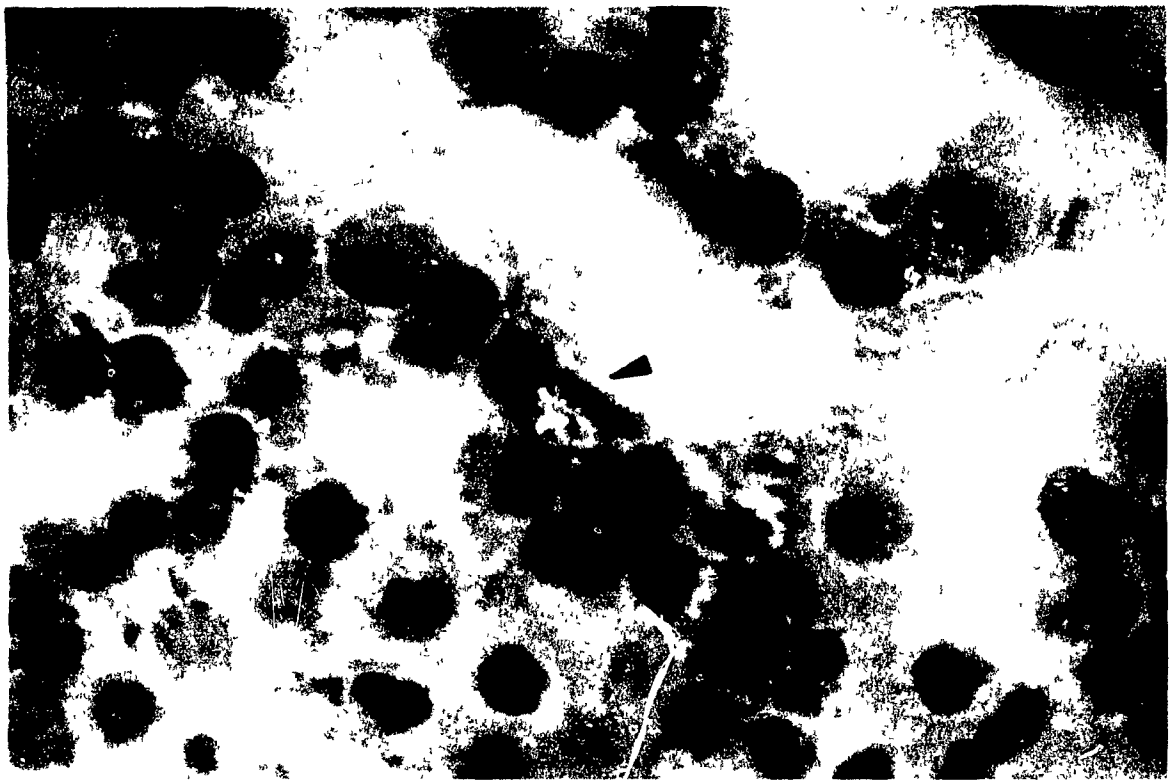
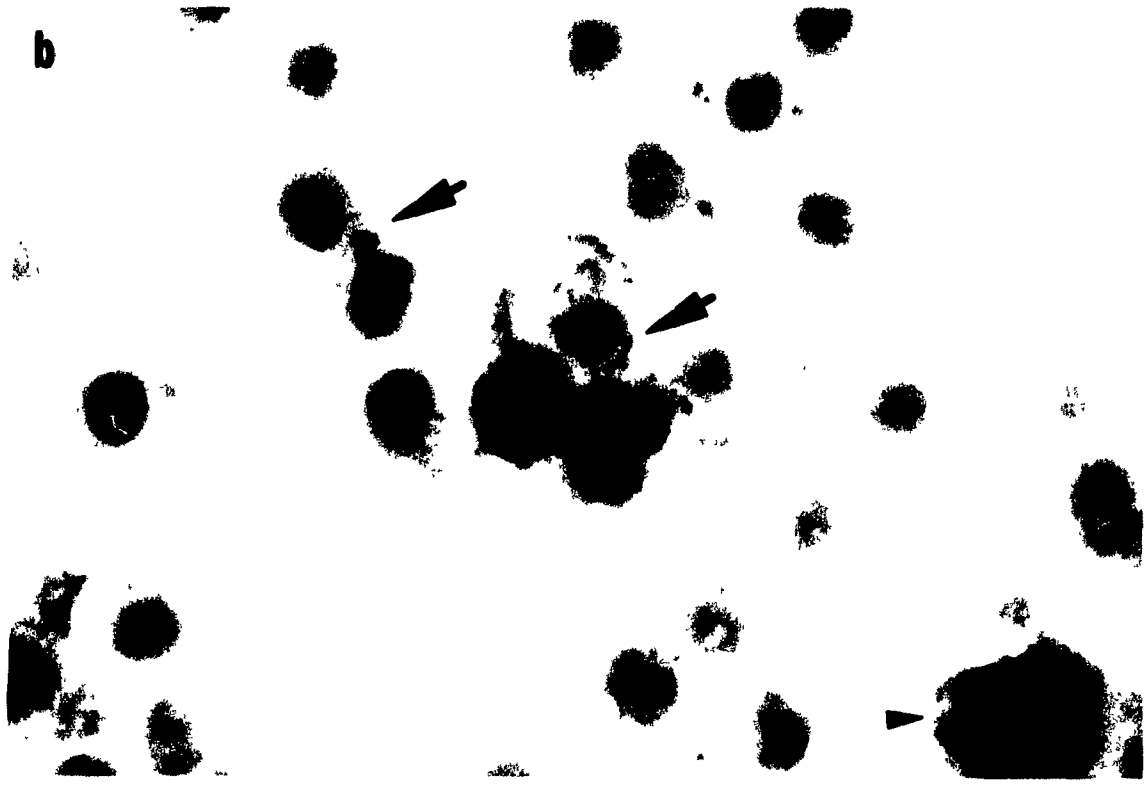


Figure 3.20. Ubiquitin-rich peritoneal cells harvested from 3 weeks post-infected C57 mice were cultured for 4 days with apo-SAA, and stained with thioflavin S or biotin-strepavidin-peroxidase method using rabbit anti-mouse AA IgG antibody; note both extracellular (*arrow*) and possibly intracellular (*arrowhead*) thioflavin S sites (**a**) and similar brown color immunoreactive sites indicative of AA (**b**); magnification of X1000.


a


b

TABLES

Table 3.1. Amyloid enhancing factor (AEF) activity of crude murine AEF and the electro-eluted peptides (EP-AEF-a, EP-AEF-b, EP-AEF-c) obtained from AEF-positive Sephacryl S-200 HR chromatography F2 fraction (see Fig. 3.1). F2 fraction was electrophoresed and the electro-eluted peptides (approximately 100 μg protein/mouse) along with crude AEF were assayed for AEF activity. Mice received subcutaneously 0.5 ml of either 1 injection of 2% or 4 injections of 1% $AgNO_3$.

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

* one mouse died
** containing 10 mM $CaCl_2$ and 10 mM dithiothreitol (DTT).
*** phosphate buffer saline, pH 7.4

Table 3.2. Results from control experiments in which mice were injected with ubiqutin alone, 4 daily subcutaneous (s.c) injections with 1 or 2% $AgNO_3$ (0.5 ml) alone or bovine serum albumin, human hemoglobin or azocasein in conjunction with 4 daily s.c injections with 0.5 ml of 1% $AgNO_3$.

Group	Treatment (number)	% and (number) of $AgNO_3$ injections	Mice sacrificed	Mice (+)ve for AA/mice examined	% AA (+)ve follicles
1.	M[illegible]e UB (50 μg; ip)	None	120 hr	0/12	-
2.	PBS, Ca^{++} (10mM)	1(4)	120 hr	0/8	-
3.	PBS, Ca^{++} (10mM)	2(4)	120 hr	0/8	-
4.	Bovine serum albumin (10 mg; ip)	1(4)	120 hr	0/4	-
5.	Human hemoglobin (10 mg; ip)	1(4)	120 hr	0/4	-
6.	Azocasein (100 μg, iv)	1(4)	120 hr	0/8	-

Table 3.3. Amyloid enhancing factor (AEF) activity of fractions obtained from CNBr activated Sepharose gel conjugated with anti-ubiquitin IgG antibody and incubated with crude AEF (flow through and 0.1 M glycine-HCl, pH 2.8 eluted fraction). The eluted fraction was fractionated on a Sephacryl S-200 HR gel column to obtain P1 and P2 fractions (for details see materials and methods). These fractions were injected intraperitoneally in conjunction with 0.5 ml of 2% $AgNO_3$ subcutaneously.

Group	Immuno-affinity purified Amyloid enhancing factor	Diluent	Number and % of $AgNO_3$ injection	Mice sacrificed	Number of mice (+)ve for AA/# number examined	% of splenic follicles (+)ve for AA	Grading of AA
1	Immuno-affinity eluted ubiquitin and adducts (0.1 mg; ip)	PBS, Ca^{2+}**	1; 2%	48 hr	4/6	54.1 ± 32.3	1+ to 3+
2	Immuno-affinity eluted ubiquitin and adducts (0.1 mg; ip)	PBS, Ca^{2+}	---	48 hr	0/4	---	---
3	Adducts (P1) separated by gel filtration after affinity-elution						
	10 µg; ip	PBS, Ca^{2+}	1; 2%	48 hr	2/6	14.4 ± 10.2	± to 1+
	100 µg; ip	PBS, Ca^{2+}	1; 2%	48 hr	6/6	77.5 ± 15.6	1+ to 2+
4	Ubiquitin (P2) separted by gel filtration after affinity-elution						
	10 µg; ip	PBS, Ca^{2+}	1; 2%	48 hr	3/5*	50.5 ± 11.5	± to 1+
	100 µg; ip	PBS, Ca^{2+}	1; 2%	48 hr	5/5	82.8 ± 22.5	1+ to 3+

* one mouse died
** phosphate buffer saline containing 10 mM $CaCl_2$

Table 3.4. Serum amyloid A protein (SAA) response in groups of C57BL/6 mice, 16 hr after the administration of crude amyloid enhancing factor (AEF), ubiquitin, bacterial lipopolysaccaride (LPS), aqueous $AgNO_3$ or phosphate buffer saline (PBS).

Groups	Reagent	Amount injected/mouse	Number/ route of injection	Mean SAA Concentration (μg/ml)
1	1% $AgNO_3$	0.5 ml	1/sc	536.6 ± 38
2	Bacterial lipopolysaccharide	50 μg	1/ip	584 ± 129.7
3	Ubiquitin	10 mg	1/ip	2.3 ± 0.52
4	Crude AEF	0.2 mg	1/ip	1.9 ± 0.21
5	PBS	0.5 ml	1/ip	< 1.0

Table 3.5. Mouse bioassay to demonstrate amyloid enhancing factor (AEF) activity of AD brain derived ubiquitin. C57BL/6 mice intraperitoneally received either crude Alzheimer's brain extract or purified Alzheimer's brain derived ubiquitin along with multiple subcutaneous injections of 1% silver nitrate solution. Spleen sections were examined for AA deposits 24 hrs after the last silver nitrate injection.

Group	Crude brain extract or purified AEF	AEF Dosage mg/mouse	Diluent	$AgNO_3$ injections	Number of mice* positive for AA/number examined	% of splenic follicles positive for AA	AA Grading
1	whole extract	1.0	PBS	4	3/3	82.9 ± 13.8	3+
2	Purified AEF ethanol-chloroform -treated	0.02	PBS, 10 mM $CaCl_2$	4	3/3	100	3+
		0.02	PBS	4	2/3	23.2 ± 10.6	± to 2+
		0.01	PBS, 10mM $CaCl_2$	4	3/3	100	3+
3	Purified AEF (heat-treated)	0.2	PBS, 10mM $CaCl_2$	4	3/3	46.6 ± 7.3	1+ to 3+
		0.2	PBS	4	0/3	-	-
		0.01	PBS, 10mM $CaCl_2$	4	2/3	48.6 ± 10.8	1+ to 3+
4	Controls	-	PBS, $CaCl_2$	4	0/3	-	-

* AA amyloid

Table 3.6. AEF bioactivity in various recombinant ubiquitin preparations in PBS and 10 mM $CaCl_2$: Male C57BL/6 mice received intraperitoneally (i.p) 50 μg recombinant wild type ubiquitin (Rc-WT UB), or 50 μg mutagenized recombinant ubiquitin species. These mice received either 3 daily subcutaneous (s.c) injections of 0.5 ml of 1% $AgNO_3$ or 1 s.c injection of 0.5 ml of 2% $AgNO_3$. Mice in the 1% $AgNO_3$ group were sacrificed 24 hr after the last $AgNO_3$ injection, and 48 hr after in the 2% $AgNO_3$ group. Control mice received either PBS with 10 mM $CaCl_2$, 50 or 100 μg azocasein (i.p), along with 3 daily 0.5 ml s.c injections of 1% $AgNO_3$. Mice in group 8 received 50 μg Rc-WT UB only. Spleens were sectioned, stained with congo-red and graded for AA deposits.

Group	Treatment	1% $AgNO_3$	AA positive mice/total	% of AA positive follicle (Mean ± SD)	2% $AgNO_3$	AA positive mice/total	% of AA positive follicles (Mean ± SD)
1	Rc-WT UB	3	3/6	96 ± 3	1	3/5	84 ± 10.9
2	Tyr-59-->Phe	3	3/4	90.4 ± 17.4	1	3/3	95 ± 10
3	Phe-4-->Cys; Glu-64-->Cys	3	2/4	97.3	1	ND***	
4	Leu-73-->Pro	3	2/4*	95.5	1	3/3	70 ± 42
5	His-68-->Lys; Tyr-59-->Phe	3	3/4	93.7 ± 5.6	1	4/4	97 ± 4.6
6	Gly-76-->Ala	3	0/4	N**	1	0/4	N
7	Lys-48-->Arg	3	1/4	98.5	1	0/4	N
8	Rc-WT UB	-	0/4	N	-	0/4	N
9	PBS + 10 mM $CaCl_2$	3	0/4	N	1	0/4	N
10	Azocasein (50 µg)	3	0/4	N	1	0/6	N
11	Azocasein (100 µg)	3	0/4	N	1	0/5	N

*2 mice died

**negative for splenic amyloid (AA)

***not determined

Table 3.7. Serum amyloid A (SAA) protein response to recombinant ubiquitin (50 μg/mouse, i.p), bacterial lipopolysaccharide (LPS, 50 μg/mouse, i.p) or 1% $AgNO_3$ (0.5 ml, 1% s.c), or PBS (phosphate buffer saline (1 ml, i.p.) as determined by enzyme linked immunosorbent assay (ELISA).

Group	Treatment	SAA (µg/ml) mean ±SD
1	Rc-WT UB	7.01*
2	Tyr-59-->Phe	6.23 ± 2.2
3	Phe-4-->Cys; Glu-64-->Cys	7.65 ± 3.5
4	Leu-73-->Pro	12.0 ± 3.8
5	His-68-->Lys; Tyr-59-->Phe	10.5 ± 5.6
6	Gly-76-->Ala	11.1*
7	Lys-48-->Arg	8.4 ± 1.9
8	1% $AgNO_3$	498 ± 81.5
9	LPS (50 µg; ip)	584.3 ± 129.7
10	PBS	< 1.0

*Detectable SAA in 2 of 3 sera (mean value)

DISCUSSION

Neither the chemical nature of AEF nor its role in amyloidosis is fully known. It was, however, recently proposed that AEF might be "a simple substance rather than a species specific complex", and "a common denominator playing a key role in the deposition phase of amyloidogenesis in different forms of amyloid" (Varga et al. 1986). Here we provide evidence in support of the above concept that ubiquitin purified from AD brain and murine amyloidotic tissues possesses AEF activity. This claim is based on (a) the amino acid homology between murine and AD tissue derived homogeneous AEF and ubiquitin (Fig. 3.1, 3.5), (b) their identical electrophoretic, immunochemical and in vivo pathophysiological properties (Figs. 3.1B, 3.3, 3.5), (c) immunoaffinity-purification of AEF positive monomeric ubiquitin and its large molecular weight adducts from crude murine AEF using monospecific RABU conjugated to Sepharose gel (Figs. 3.2, 3.4, Table 3.3), and finally abolition of AEF activity in crude murine AEF (Fig. 3.8). The demonstration that recombinant ubiquitin has AEF activity analogous to that of crude AEF or tissue derived Ub, and this activity and in vitro amyloidogenesis are abolished by treatment with RABU (Fig. 3.9) further strengthens my claim about the chemical nature of AEF. Thus ubiquitin seems to fulfil the predictions proposed for AEF: that it is a "simple" peptide found universally in all eukaryotic cells and may very well be "a common denominator" and possibly a pathogenetic link in "different forms of amyloid" (Varga et al. 1986). The data presented in this thesis unambiguously show that monomeric ubiquitin purified from two chemically distinct amyloid laden tissues (AD brain and murine tissues) possess AEF activity. Although AEF has also been demonstrated in extracts from tissues laden with AL and AF types of amyloid (Varga et al. 1986), it remains to be seen whether the AEF activity in such samples is associated with ubiquitin as well.

Initially we used, as before (Ali-Khan et al. 1988b, Alizadeh-Khiavi et al. 1990a), 4 daily s.c. injections of 0.5 ml of 1% $AgNO_3$ to identify AEF activity in the electro-eluted fractions from SDS-PAGE gel (Table 3.1). Control mice receiving either four inflammatory stimuli only (Table 3.2), or EP-AEF-a only (Table 3.1) failed to induce amyloid in mice. These formed the basis for the present discovery of AEF activity in ubiquitin. Also as shown in Table 3.2, regardless of whether the control mice were given 4 daily s.c. injections of 0.5 ml of 1 or 2% $AgNO_3$ only, or human hemoglobin, bovine serum albumin or azocasein in conjunction with 1% $AgNO_3$, splenic AA deposition did not occur. These results are in accord with previous studies in which 7 to 10 daily s.c. injections with azocasein (0.5 ml of 7%) or $AgNO_3$ (0.5 ml of 2%) were required to induce amyloid in mice (Axelrad et al. 1982, Kisilevsky et al. 1977). Subsequently, using the conventional bioassay technique (Axelrad et al. 1982), we demonstrated dose-dependent AEF activity in the tissue derived monomeric ubiquitin and its adducts (Fig. 3.3, Table 3.3). Thus, these results clearly show that ubiquitin, purified by the ethanol-chloroform method (Matsumoto et al. 1984) and unexposed to heat-treatment, contains potent AEF activity.

We next addressed an important question regarding the presumed heterogeneity of AEF: should there be one or more than one species of AEF. Previous bioassay studies, using exclusion chromatography fractions from crude AEF, predicted AEF to be a heterogeneous moiety ranging in molecular weight from 12 to >100 K (Ali-Khan et al. 1988b, Axelrad et al. 1982, Alizadeh-Khiavi and Ali-Khan 1988, Niewold et al. 1987, Yokota et al. 1989a). Due to the lack of an anti-AEF antibody the question whether AEF is a heterogeneous moiety has remained unresolved. This was partly resolved by immunoblotting and affinity purification methods using RABU (Figs. 3.2, 3.4). The affinity purified adducts and monomeric ubiquitin tested positive for AEF activity (Fig. 3.4, Table 3.3). Also it is important to note that similar to the apparent tendency of AEF to bind to particulates (Axelrad et al. 1982), AA microfibrils (Niewold et al. 1987) and

nucleoproteins (Hardt and Hellung-Larsen 1972), ubiquitin also binds to AA amyloid protein (Chronopoulos et al. 1990a & 1990b, Alizadeh-Khiavi et al. 1990a), forms metabolically stable conjugates with histones, and with a variety of short-lived intracellular regulatory proteins which become targets of ubiquitin-mediated proteolysis (Rechsteiner 1989, Monia et al. 1990, Haas and Bright 1985). These characteristics of ubiquitin correspond well with that of AEF. In another approach I used RABU in a functional assay to neutralize AEF activity, similar to that used for blocking the protease-like activity of ubiquitin (Fried et al. 1987, Vincent et al. 1990), or choline uptake by ubiquitin-protein conjugate on the central cortical synaptosomal surface (Meyer et al. 1986). RABU in a dose-dependent manner abolished AEF activity in the crude AEF (Fig. 3.8), recombinant ubiquitin (Fig. 3.9), and abolished AA amyloid generation in in vitro (see results section 3.12). Taken together these results are at variance with the predicted concept of AEF heterogeneity. If it were the case, the blocking effect of RABU against the crude AEF would have been partial. Thus, we suggest that the large molecular weight AEF, as reported previously (Ali-Khan et al. 1988b, Axelrad et al. 1982, Alizadeh-Khiavi and Ali-Khan 1988, Niewold et al. 1987, Yokota et al. 1989a), may be the ubiquitin adducts as shown here (Fig. 3.2).

Experiments described in this thesis also distinctly demonstrate the presence of AEF bioactivity in recombinant ubiquitin when concomitantly administered into mice with either 3 daily 1% s.c. $AgNO_3$ or a single 2% s.c. $AgNO_3$ (Table 3.6). Meanwhile Rc-WT UB alone did not induce accelerated amyloidogenesis. Neutralization of AEF activity in Rc-WT UB with anti-ubiquitin antibody (Fig. 3.9) further confirms that AEF activity indeed lies within ubiquitin. Moreover, neither azocasein (an unrelated protein), nor PBS and Ca^{2+} (Table 3.2, groups 2,3,6; Table 3.5, group4; Table 3.6, groups 9-11) were capable of inducing accelerated amyloidogenesis (Alizadeh-Khiavi et al. 1990a & 1990b). Availability of various mutagenized Ub (created for other purposes) and their use in the AEF assay (table 3.6) offer some insight into the possible molecular pathogenesis of AA amyloidosis.

It is now widely accepted that carboxyl terminal amino acid residue (Gly 76) of Ub is involved in the conjugation of ubiquitin to target proteins and their ATP dependent proteolysis (Ecker et al. 1987, Rechsteiner 1987, Wilkinson 1988). Ubiquitin lacking its C-terminal dipeptide neither undergoes conjugation nor supports the proteolysis of protein substrates (Wilkinson and Andhya 1981). Gly76 of Ub is activated prior to its conjugate formation and contribution of its carboxyl group to the isopeptide bond (Hershko and Heller 1985). Interestingly the Ub with Ala to Gly substitution (table 3.6) failed to demonstrate AEF activity. Based on our proposed data, we can only speculate at this time that lack of Gly76 may have inhibited the binding of Ub to SAA and its further processing into AA. Thus the critical first step seems to be to examine the nature of the SAA-Ub interaction in in vitro. Furthermore, ubiquitin's lysine-48 residue, which is the site involved in the formation of multiubiquitin chains (Chau et al. 1989), may play an essential role in relation to AEF activity (Table 3.6; group 7). Lysine 48 has been shown to involve in ubiquitin-ubiquitin bindings (ubiquitin trees) and polyubiquitination is suggested as a prerequisite for proteolysis of substrate proteins (Chau et al. 1989, Monia et al. 1989). Previous studies involving chemical modifications or mutagenesis of other lysine residues (7 altogether) have provided some evidence in support for a minor role that these residues may play in substrate degradation (Gregori et al. 1990). This may also account for the presence of a minor AEF activity in Ub with Lys48 substitution. On the other hand, disulphide bond formation does not interfere with amyloidogenic function of ubiquitin, since the disulphide mutant (Table 3.6; group 3) did not show any decrease in AEF activity. Other mutations including His-68->Lys Tyr-59->Phe (Table 3.6; group 5), Leu-73->Prol (group 4), and Tyr-59->Phe (group 2) did not have any major effect in AEF activity of these mutant proteins indicating the unimportant role of these internal residues in amyloidogenesis by ubiquitin.

We, like others (Axelrad et al. 1982, Deal et al. 1982, Sipe et al. 1986, Hebert and Gervais 1990), were unable to detect either amyloidogenesis or SAA elevations in mice exposed to either crude AEF or ubiquitin (Table 3.1, group 3; Table 3.4). These events, however, occur in response to or in concert with $AgNO_3$ injection. Thus it does seem that ubiquitin may play a critical but as yet undefined role in amyloidogenesis during high levels of circulating SAA (Gertz et al. 1985, Meek et al. 1986, Sipe at al. 1978). The evidence presented in Fig. 3.7, 3.12-15 clearly suggest that AEF-induced or AHC-induced murine AA type amyloid fibrils and ubiquitin interact with each other during amyloidogenesis. Both ubiquitin (Alizadeh-Khiavi et al. 1990a, Chronopoulos et al. 1990b) and SAA (Du and Ali-Khan 1990, Chronopoulos et al. 1990b, Meek et al. 1989, Miura et al. 1990) codeposit in splenic PFAs which are believed to be one of the important anatomical sites for SAA clearance (Kisilevsky 1983). Therefore, we propose an in vivo working model for the pathogenesis of AA amyloidosis in the AHC infected mice. Neutrophils, macrophages and reticuloendothelial (RE) cells have been associated with the formation of amyloid (Fig. 3.10,11) (Glenner 1980, Kisilevsky 1983, Kisilevsky et al. 1987, Smetana 1927, Fuks and Zucker-Franklin 1985, Shirahama and Cohen 1975, Alkarmi and Ali-Khan 1984, Wisniewski et al. 1989). These cells, during inflammatory stress conditions contain high levels of ubiquitin (Fig. 3.10,11; Alizadeh-Khiavi et al. 1990b, Chronopoulos et al. 1990a & 1990b), or as shown previously AEF (Abankwa and Ali-Khan 1988b, Alizadeh-Khiavi and Ali-Khan 1988, Shirahama et al. 1990). Recently, AEF-loaded macrophages were implicated in the processing of SAA to AA (Shirahama et al. 1990). Similar results obtained with AHC-induced peritoneal macrophages (Fig. 3.18-20) suggest that AEF/ubiquitin released from such cells either alone (Fried et al. 1987, Vincent and Davies 1990) or in concert with other extracellularly released proteases (Lowe et al. 1990, Ganoth et al. 1988, Eytan et al. 1989), might proteolyse SAA into AA. The critical role of ubiquitin in amyloidogenesis is emphasized by the fact that RABU can inhibit the in vitro processing of SAA into AA. Both intact or partially degraded SAA have been

demonstrated in AA deposition sites (Du and Ali-Khan 1990, Chronopoulos et al. 1990b, Meek et al. 1990, Miura et al. 1990) and we have shown binding of ubiquitin to murine AA in this thesis (Fig. 3.9, 3.10, 3.13) and elsewhere (Chronopoulos et. al. 1990a & 1990b). This interaction appears to be of non-covalent nature (Chronopoulos et al. 1990a & 1990b). In effect, a physiological role has been suggested for ubiquitin found associated with neurofibrillary tangles (Grundke-Iqbal et al. 1989, Perry et al. 1989, Lowe et al. 1990a, Ivy et al. 1989), scrapie amyloid plaques (Lowe et al. 1990b), diffuse to perivascular plaques in Alzheimer's disease (Suenaga et al. 1990), and to several other fibrillar inclusions (Lowe et al. 1990a).

Intracellularly, ubiquitin is found either free in a monomeric form, or linked via its COOH-terminal glycine residue to ϵ-NH_2 groups of lysine residues of a variety of cytoplasmic, nuclear and cell surface proteins (Rechsteiner 1989, Minia et al. 1990). During conditions of cellular stress (Rechsteiner 1989, Monia et al. 1990) or as shown in motor neuron disease (Heggie et al. 1989), both the transcription and expression of ubiquitin increases significantly. Specificity of ubiquitin to bind and to proteolyze particularly short-lived proteins or structurally abnormal proteins through ATP-dependent mechanism, have been well established (Ganoth et al. 1988, Eytan et al. 1989). Nevertheless, the precise role of ubiquitin in the formation of ubiquitinated fibrillar inclusions or its association with murine AA and other amyloids is unclear at this time, although evidence that ubiquitin/AEF may act against proteolytic substrates similar to serine proteases is provocative (Fried et al. 1987). Besides ubiquitin, several other host components such as elastase (Skinner et al. 1986), α1-antichymotrypsin (Abraham et al. 1988), amyloid P component (Skinner et al. 1982), and sulphated glycosaminoglycans (Kisilevsky 1987), bind to amyloid. As yet none of these are known to possess AEF activity (Axelrad et al. 1982, Snow et al. 1987), although they have been implicated to play a physiological role in amyloidogenesis. Given that ubiquitin has AEF activity, it binds non-covalently to murine AA and both leukocytes and RE

cells get "loaded" with ubiquitin during amyloidogenesis, a circumstance analogous to that of AEF-loaded macrophages (Shirahama et al. 1990), it is reasonable to propose that ubiquitin may play a crucial role in amyloidogenesis.

Of further interest, is the stimulatory role of calcium (Ca^{2+}) in ubiquitin-mediated amyloidogenesis (Table 3.i) or proteolytic activity (Fried et al. 1987). High concentrations of Ca^{2+} are found in AA amyloid deposits (Kula et al. 1977), and all the known SAA and AA sequences (Yamamoto and Migita 1985, McCubbin et al. 1988) contain a common tetrapeptide sequence Gly^{48}-Pro^{49}-Gly^{50}-Gly^{51} which is homologous to the main Ca^{2+} binding site, Gly^{30}-Xaa^{31}-Gly^{32}-Gly^{33}, of bovine phospholipase A2 (Turnell et al. 1986, Dennis 1983). Recent studies on mouse SAA_1 and SAA_2 suggest that both SAA species bind Ca^{2+} (McCubbin et al. 1988). Thus it seems likely that during the preamyloid phase ubiquitin, requiring Ca^{2+} for its catalytic/proteolytic activity (Fried et al. 1987), gets sequestered in the extracellular matrix with a Ca^{2+} binding precursor protein of amyloid. Whether convergence of these disparate factors are fortuitous or have a biological significance in amyloidogenesis remains to be elucidated.

Ultrastructural studies on amyloidotic tissues have led to two theories explaining the possible mechanisms of amyloidogenesis. According to the intracellular model, amyloid fibrils are formed inside the lysosomes of reticuloendothelial (RE) cells (Shirahama and Cohen 1975, Ishihara and Uchino 1975) or in an organelle of macrophage/ microglia similar to that of smooth endoplasmic reticulum (Wisniewski et al. 1990). According to the second model, extracellular processing of SAA and polymerization of AA occurs in conjunction with membrane associated enzymes of macrophages (Lavie et al. 1980, Zucker-Franklin and Fuks 1986). Evidence for both these circumstances is based on ultrastructural studies. In light of these observations, the concept of "fibril-AEF" (Niewold et al. 1986 & 1987), a situation analogous to that of ubiquitinated murine AA (Fig. 3-7, 3.12-15; Chronopolous et al. 1990a & 1990b, Alizadeh-Khiavi et al. 1990a)

is of considerable interest. "Fibril-AEF", on passive transfer into mice behaves like AEF, has an estimated molecular weight of 12 K (estimated by exclusion chromatography) and is believed to represent tissue AEF bound to AA microfibrils (Niewold et al. 1986, 1987). It was postulated that "fibril-AEF" may act as nucleants in accelerating their own production. An analogous mechanism has been proposed in the induction of the transmissible and the non-transmissible amyloidosis of the brain (Guiroy and Gajdusek 1988). The ubiquitinated AA fibrils seem to correspond to these nucleants. Also ubiquitin deposits have been demonstrated in some compact Alzheimer plaques, in most perivascular plaques, in hippocampal pyramidal neurons showing granulovacuolar degeneration (primordial stage of a senile plaque and in prion plaques in Kuru) (Suenega et al. 1990a and 1990b, Lowe et al. 1988 and 1990a). Thus it seems safe to predict that regardless of whether amyloidosis formed intracellularly or extracellularly, its ubiquitination may prove to be a key link to understanding the pathogenesis of amyloidosis.

The findings of increased AEF activity in crude AD brain extract (Ali-Khan et al. 1988b) and identification of AEF activity in AD-brain ubiquitin (Alizadeh-Khiavi et al. 199a) offers me an opportunity to explore the possible role of Ub in AD. Amyloidosis has often be ascribed to an age-dependent change, probably a sequela of functional decline in normal physiology. This is evidenced by the fact that amyloid deposition in the brain increases with age even in normal people. However to the extent that A4 protein deposition involves the limbic and associative cortices in AD and Down's syndrome patients, it clearly indicates a pathological process. At a minimum, it may involve at least two parallel events: an abnormal synthesis and turn-over of precursor protein of A4 amyloid and second, an increased synthesis of ubiquitin/AEF. Both these events have now been demonstrated to occur during normal aging. Preliminary studies indicate a dramatic increase in mRNA levels of precursor protein of A4 in senescent cultured fibroblasts (Adler et al. 1991). Similarly, heavily ubiquitinated deposits have been demonstrated in the

normal senescent brain (Pappolla et al. 1989). What then triggers Alzheimer-associated changes in the brain? Based on the data presented in this thesis, I propose a tentative mechanism which may in part help to understand the pathogenesis of Alzheimer's disease. It is now well established that glial proliferation accompanies AD pathological changes (Wisniewski et al. 1989 and 1990, Itagaki et al. 1989). Microglia which structurally, physiologically and functionally resemble cells of the monocytoid/ macrophage series (Zucker-Franklin et al. 1987, Giulian et al. 1986) and astrocytes infiltrate edges of virtually all senile plaques. These events appear to be analogous to those of activated monocytoid cells infiltrating in the site of trauma. Since IL-1 has been shown to induce increased transcription and expression of ABPP genes (Goldgaber et al. 1989, Griffin et al. 1989), as in the case of SAA by murine hepatocytes and other cells (Meek et al. 1989), therefore IL-1 released by activated microglia (Zucker -Franklin et al. 1987, Giulian et al. 1986) should conceivably induce increased expression of ABPP by the neurons and thus render "abnormal" accumulation of ABPP in the neuropil. I (Fig. 3.10,11) and others (Chronopoulos et al. 1990a and 1990b) have shown increased synthesis and/ or reorganization of cytosolic Ub in activated macrophages, RE cells and PMNs during inflammatory stress conditions. It is conceivable that similar events might occur in the activated microglia. More recently direct evidence has been found in support of increased transcription of a gene coding for a polyprotein of ubiquitin and the generation of ubiquitinated filamentous inclusion bodies in neurons in motor neuron disease (Heggie 1989). Lowe et al. (1990b) also described a tentative role for ubiquitin carboxyl-terminal hydrolases (PGP 9.5) in the biogenesis of ubiquitinated fibrillar inclusion bodies in neuronal and non-neuronal tissues. Thus, at least superficially, in both AA amyloidosis and AD, there appears to be a parallel increase in one of the stress proteins (Ub) and a short-lived protein (ABPP or SAA). To my knowledge no biological component, except ubiquitin, with such a biological activity (AEF activity) has yet been described.

To summarize, the present study brings into focus the following: first, convergence, during acute inflammation, of two stress-related phenomena - the acute phase response and the heat-shock or stress response; and second, the involvement of their respective expressed reactants, SAA and ubiquitin, in the induction of amyloidosis. Induction of stress response (increased level of ubiquitin; Fig. 3.14, Alizadeh-Khiavi et al. 1990b, Chronopoulos et al. 1990a & 1990b) during an acute phase response had not been recognized previously (Permutter 1988). Together they add a new dimension to the possible role of stress (Hall et al. 1960, Page and Glenner 1972, Cowan and Johnson 1970) i.e ubiquitin in AA amyloidogenesis. Nonetheless, both ubiquitin and SAA responses appear to be mutually exclusive events, although in the AHC-infected mice inflammatory stress conditions act as the common denominator. How the sentinel-like role of cytosolic ubiquitin (Rechsteiner 1987, Monia et al. 1990, Eytan et al. 1989, Ivy et al. 1989) is altered in the extracellular matrix appears to be essential to the understanding of the pathogenesis of amyloidosis.

CONCLUSION

The present thesis provides data supporting the claim that AEF activity is associated with ubiquitin. This claim is based on the following:

a) The partial amino acid sequence homologies between ubiquitin and the homogeneous AEF positive peptides purified from amyloidotic murine tissue and AD brain extracts.

b) The in vivo amyloid accelerating properties of purified murine or AD ubiquitin, as well as recombinant wild type ubiquitin.

c) The presence of AEF activity in immunoaffinity purified ubiquitin and some of its putative adducts.

d) The immunoreactivity of peptides purified from murine or AD tissues, with anti-bovine ubiquitin IgG antibody in Western immunoblotting.

e) The abolition of the in vivo amyloid accelerating properties of crude mouse AEF and recombinant wild type ubiquitin with anti-bovine ubiquitin IgG antibody.

f) An increased anti-bovine ubiquitin IgG antibody immunoreactivity of inflammatory murine leukocytes during the preamyloid and amyloid deposition phases; this phenomenon appears to be analogous to that of AEF-loaded leukocytes observed during amyloidogenesis with the murine model of inflammation associated amyloidosis.

g) The in vitro generation of amyloid deposits by AEF-loaded/ ubiquitin-rich murine peritoneal macrophages, and abolition of the amyloidogenesis by anti-ubiquitin antibody.

h) The immunoelectron microscopic identification of ubiquitin-rich compartments in inflammation associated peritoneal cells in mice.

i) The colocalization of amyloid AA fibrils with ubiquitin in splenic PFAs in accelerated model of amyloidosis or AHC-infected mice, demonstrated by immunohistochemistry and immunoelectron microscopy.

j) And finally, based on the identification of AEF activity in ubiquitin, a hypothesis is proposed for the pathogenesis of amyloidosis. It describes interaction between SAA (the precursor protein of AA amyloid) and ubiquitin, both expressed during inflammatory stress conditions, as the principal prerequisite events in amyloidogenesis.

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