PATHOLOGICAL AND IMMUNOCHEMICAL CHARACTERIZATION
OF SECONDARY AMYLOIDOSIS IN CHRONIC
MURINE ALVEOLAR HYDATIDOSIS.

by

Tarif Osama Al Karmi

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Department of Microbiology
and Immunology,
McGill University,
Montreal, Quebec.

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Suggested short title:
Alveolar hydatidosis and secondary amyloidosis.
DEDICATION

I would like to dedicate this thesis to my beloved parents and to Tahani, Naser, Rana, Raed and Amer.
PATHOLOGICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF SECONDARY AMYLOIDOSIS IN CHRONIC MURINE ALVEOLAR HYDATIDOSIS

Ph.D. Tarif Osama Al Karmi Microbiology & Immunology.

Induction of amyloid and associated pathological changes induced by alveolar hydatid cysts (AHC), the larval stage of Echinococcus multilocularis, were studied in Balb/c, C57BL/6J, CBA and A/JAX strains of mice. The onset and incidence of amyloidosis in these four strains corresponded with the intensity of hepatic inflammation and not with the load of parasite biomass. The spleens of AHC-infected mice were more sensitive to amyloid deposition than livers or kidneys. Splenic amyloid deposits appeared at 6 weeks post-infection (p.i.) in Balb/c and C57BL/6J mice and at 8 and 12 weeks p.i. in CBA and A/JAX mice. Amyloid proteins were found to be resistant to potassium permanganate and trypsin treatments. Rabbit antisera raised against mouse AA-amyloid protein (Azocasein induced) was shown to cross-react with AHC-induced amyloid (AHCA). The immunological cross-reactivity was determined by three different assays: immunoperoxidase, indirect immunofluorescence and Ouchterlony tests. In ultra thin sections of spleens, livers or kidneys from AHC-infected mice, amyloid fibrils appeared to be non branching and measured 8-12 nm. in diameter. In order to determine the chemical nature of AHCA, it was extracted from the tissues of C57BL/6J mice at 12 weeks p.i., either in water or 6 M acid-urea buffer. The AHCA was then purified by gel filtration on Sephadex G-100 and G-50. By using SDS-PAGE and isoelectric focusing (pI),
the AHCA protein showed a molecular weight (Mw) of 8,000 daltons and a pI value of 5.3. Azocasein-induced and purified amyloid protein from C57BL/6J mice had a similar MW but a pI value of 5.8. The amino acid composition of purified AHCA presented a closer relationship of AHCA to senesence-induced (age associated) amyloid protein than to AA-amyloid. The role of AHC-derived factors in the pathogenesis of secondary amyloidosis was studied in vitro and in vivo. AHC-antigens were purified by affinity chromatography and by gel filtration on Sephacryl S-300. Purified AHC-antigens were cytotoxic and chemotactic when tested in vivo and phlogistic when introduced intradermally or intraperitoneally in mice. Furthermore, AHC-antigens were amyloidogenic. The injection of 1 mg i.p. in mice induced amyloid deposition after 96 hours in their spleens. In Balb/c, C57BL/6J and A/JAX mice, amyloid deposition was correlated with the appearance of an amyloid enhancing-like factor (AHC-AEF) in the spleens. AHC-AEF appeared just prior to amyloid deposition. The passive transfer of AHC-AEF to allogeneic or syngeneic mice induced amyloid formation in the spleens within 16-48 hours when followed by silver nitrate administration subcutaneously, or after 1 week when given concomitantly to AHC-infected mice. Partial purification of AHC-AEF shows that it is soluble in physiologic media, its Mw ranges between 30 to 100 K daltons and its activity can be pelleted by ultracentrifugation. AHC-AEF is resistant to DNAase, RNAase, pepsin and trypsin treatments. However it lost its activity when incubated in a boiling water bath for 1 hour.
La production d'amylose et les changements pathologiques induits par les kystes hydamiques alvéolaires (KHA) dus au stade larvaire d'Echococcus multilocularis, ont été étudiés chez les souris de souche Balb/c, C57BL/6J, CBA et A/JAX. L'apparition et l'incidence de l'amylose chez ces quatre souches de souris étaient reliées à l'intensité de l'inflammation hépatique et non à la charge parasitaire. Les dépots d'amylose étaient plus importants dans la rate des souris AHC que dans leur foie ou leurs reins. Les dépots spléniqnes d'amylose sont apparus 6 semaines après l'infection chez les souris Balb/c et C57BL/6J alors que cet intervalle était respectivement de 8 à 12 semaines chez les souris CBA et A/JAX. Les dépots protéiques d'amylose étaient résistants au traitement par le permanganate de potassium et par la trypsine. Des antisera de lapin dirigés contre la protéine amyloide AA avaient une haute affinité pour l'amylose induite par KAH (AKAH) d'après des tests d'immunoperoxydase et d'immunofluorescence. Sur des coupes ultrafinnes de rates, foie ou reins de souris infectées par le KAH le diamètre des fibres d'amylose était de 8 à 12 nm. L'AKAH a été extraite des tissus de souris C57BL/6J 12 semaines après l'infection, en utilisant, soit de l'eau soit un tampon acide-urée 6M. L'AKAH a été ensuite purifiée par filtration sur gel de Sephadex G-100 et G-50. Par électrophorèse en gel de polyacrylamide et par isofocalisation, la protéine amyloide avait un poids moléculaire (PM) de 8 000 et un point isoelectrique (pI) de 5.3. La protéine amyloide induite par l'azocaséine chez la souris C57BL/6J avait un PM similaire et un pI de 5.8. La composition en acides aminés de l'AKAH purifiée ressemblait plus à celle de l'amylose associée à la senescence qu'à celle de l'amylose AA. Les antigènes des KAH furent purifiés par chromatographie d'affinité et par filtration sur gel de Sephacyr S-300. Les antigènes purifiés de KAH étaient cytotoxiques et chimiotactiques selon des tests in vivo et phlogogènes après administration intradermique et intrapéritonale. De plus les antigènes de KAH étaient amylogènes. L'injection d'un mg d'antigène i.p. était suivie 96 heures plus tard de déposition
d'amylase dans la rate. Chez les souris Balb/c, C57BL/6J et A/AJAX le dépôt d'amylase s'accompagnait de l'apparition d'une substance de type Facteur d'Augmentation de l'Amylose (KAH-FAA) dans la rate, juste avant le dépôt d'amylase. Le transfert pas-sif de KAH-FAA à des souris allogéniques ou syngéniques s'ac-compagnait de dépôt splénique d'amylase 16 à 48 heures plus tard, après administration sous-cutanée de nitrate d'argent, ou une semaine plus tard après infection par KAH. Une purifi-cation partielle du KAH-FAA a montré qu'il est soluble dans les milieux physiologiques, que son PM est compris entre 30 et 100 Kdaltons et que son activité est sédimentable par ultra-centrifugation. Le KAH-FAA est résistant à la ADNase, ARNase, la pepsine et la trypsine. Néammoins, son activité est abolie par l'ébullition à 100 degrés pendant une heure.
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I would like to express my appreciation and sincere thanks to my father and mother for their moral and financial support throughout this study and for being such wonderful and understanding parents. I would also like to express my appreciation and thanks to my research supervisor Dr. Zafer Ali-Khan for his supervision and continuous help throughout this study. My sincere thanks are also due to Dr. C. Zarkadas at Macdonald college of McGill university for his assistance and use of amino acid analysis and computer equipments. I would also like to express my gratitude to Drs. M. Baines, E.C. Chan, F. Archibald, J. Coulton, R. Siboo and all the staff at the department of microbiology for their instructive discussion and encouragement. My sincere thanks are also due to all my friends at the department: Alan Lazarus (the lizard man), P. Pannunzio (the crazy italian), U. Mcguiness, K. Glasgow, and O. Abukhres. Special thanks are also due to all my friends in the parasitology laboratory: Mike Ganon, G. Abankawa, J. Charest, P. Skalenda, Karen Eugeni and J. Cabeza for their understanding, support, and help. Finally, I would like to thank my dear friend Dr. Kazem Behbehani for his advice and encouragement, the F.C.A.C. and McGill university, the department of Microbiology and Immunology in particular for the scholarships and bursaries I have received during the period of my study.

VII
CLAIM OF ORIGINALITY

In this study the following information has been added to the literature.

(1) Characterization of the amyloidogenic potential of alveolar hydatid cyst (AHCA) in amyloid susceptible and amyloid resistant strains of mice.

(2) Effect of inoculum size and route of infection on the deposition of AHCA-induced amyloid (AHCA).

(3) Isolation, purification and physico-chemical characterization of AHCA (Mw, pI, and amino acid composition).

(4) Ultramicroscopic and immunohistochemical characterization of the amyloid protein in situ and its comparison with azocasein-induced AA-protein.

(5) Determination of the susceptibility of AHCA to KMnO₄ and trypsin treatments.

(6) Isolation and determination of the diverse biological activities of AHCA-derived pathogenetic factors: a. phlogistic. b. chemotactic. c. cytotoxic. and d. amyloidogenic.

(7) Isolation, partial purification and determination of the bioactivity of an amyloid enhancing-like factor from AHCA-infected mice.
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Echinococcus or hydatid disease is a cyclozoanotic infection caused by the organotropic larval stage of *Echinococcus granulosus*, *E. multilocularis*, *E. oligarthus* and *E. vogeli*. The larval forms, unilocular type (*E. granulosus*), the alveolar type (*E. multilocularis*) and the polycystic type (*E. oligarthus*) have been recovered from humans and are designated as hydatid cysts (Wilson and Rausch 1980). These larval forms differ from each other both structurally and in their course of infection in humans. *E. granulosus* and *E. multilocularis* are enzootic in Canada but the former is the most prevalent species (Smyth and Smyth 1963; Leiby et al. 1970; Williams et al. 1971).

**1. ALVEOLAR HYDATID DISEASE**

Alveolar hydatid disease (AHD) which is caused by alveolar hydatid cysts (AHC) is one of the most serious parasitic diseases. By the time the disease becomes clinically apparent (after several years or decades) the lesion is often inoperable and the mortality rate ranges from 50 to 70% (Webster and Cameron 1967, Mosimann 1980, Schantz et al. 1983). Although humans are unsatisfactory hosts, the course of AHC infection in humans is invariably prolonged and the clinical manifestations are quite variable. In some individuals, the course of AHC may undergo a period of latency (inactive stage) before developing into a progressing metastasizing mass while in others the AHC may proliferate indefinitely by exogenous budding and behave like a
malignant tumor (Wilson and Rausch 1980). Metastatic foci, however, develop many years probably a decade or so, after the primary infection when the host's defense mechanisms are compromised. Wilson and Rausch (1980) have questioned whether the AHC ever becomes inactive spontaneously in humans. This implies that hydatid-patients are unable to abrogate the AHC, although they may control its proliferation temporarily.

Alveolar hydatid disease is primarily confined to the northern hemisphere although sporadic human cases have been reported from India, Iran and South America (Grases and Salazar 1970; Leiby et al. 1970; Mirzoyans 1975; Aikat et al. 1978). The infection is prevalent in Central Europe, Canada, the United States, Siberia, Iceland, and northern Japan (Yamashita 1960; Matossian et al. 1977). Wilson and Rausch (1980) have recently reviewed 33 indigenous cases of AHD from North America. In the last three decades, several epidemiological surveys have been carried out to map the focus of E. multilocularis infection in Canada and the U.S.A. The presence of AHC in local rodents in Central North America (Dakotas, Minnesota, Iowa, Montana, Wyoming, Manitoba, Saskatchewan and Alberta) and Alaska is believed to represent a potential source of infection for cats and dogs which in turn can transmit the infection to humans (Leiby and Olson 1964; Leiby, 1965; Carney and Leiby 1968; Leiby et al. 1970).

There is no doubt that AHD is ill diagnosed because the symptoms are subjective and vague depending on the size and location of the proliferating AHC. In Switzerland, out of 67 AHD cases that were proven at autopsy only 18 cases were diagnosed
correctly (Webster and Cameron 1967). Even at necropsy the disease was mistaken for a malignant tumor. Therefore, screening the human population for AHD is rather premature and may lead to underestimating the seriousness of the problem. To correctly obtain a worldwide epidemiological profile of the distribution of the disease, both susceptible definitive and intermediate hosts have been included in the survey. In fact, current studies on the distribution of AHD include a wide variety of intermediate hosts (Smyth and Smyth 1964; Williams et al. 1971; Leiby et al. 1970; Matosian et al. 1977).

In Canada, the parasite was initially thought to be confined to the Arctic (Cameron, 1960), but recent surveys have revealed a relatively high incidence of *E. multilocularis* infections in both the definitive and intermediate hosts in British Columbia, the Northwest Territories, Saskatchewan, Alberta and Manitoba (Webster & Cameron 1967, Lubinsky & Galaugher 1969; Choquette et al. 1962; Hanatiuk, 1966). It is apparent that due to the migration of the natural hosts the parasite is spreading to more heavily populated areas to the south and into the United States (Leiby & Olson, 1964; Leiby, 1965). In 1979, Gamble et al. reported the first autochthonous human case of AHD acquired in Minnesota, USA. The spread of the disease to animals, such as field mice and house pets, which are the main source of infection is very alarming. It is estimated that this may result in an increase in the number of human AHD cases within the coming 10-20 years.

The adult *Echinococcus multilocularis* belongs to a genus
Phylum: Platyhelminths
Class: Cestoidea
Subclass: Cestoda
Order: Cyclophyllidea
Genus: Echinococcus

The adult and larva of *E. multilocularis* are morphologically distinguishable from the other species.

**Morphology, Biology and Life Cycle.**

The life cycle of the parasite is shown in Fig. 0-1. Essentially the adult *E. multilocularis* lives in the small intestine of the definitive hosts, the arctic fox, *Alopex lagopus* (Rausch, 1968) and the red fox, *Vulpes vulpes*. Other animals such as coyotes, dogs and cats may also serve as definitive hosts. The adult tapeworm measures 1.2–3.7 mm in length and consists of a scolex (head), a short neck, and immature, mature and gravid proglottids. The scolex contains 2 pairs of suckers and a non-retractable armed rostellum, bearing two rows of 30–36 hooklets. The suckers and hooks are used for attachment to the intestinal epithelium of the host. When the eggs mature, the gravid proglottid detaches and ruptures, releasing the ova containing the oncosphere (hexacanth embryo). Infection occurs when embryonated ova are accidentally swallowed by the intermediate host (man and several other species of small rodents such as
The life cycle of *Echinococcus multilocularis* (Leuckart, 1863).

(A) **Definitive host of the parasite (fox, dog and cat).** Sexually mature worms develop in the small intestine.

(B) **Intermediate host (mouse, vole, rodents in general and, accidentally, man).** Only the larval stage of the parasite, the alveolar hydatid cyst, develops in the intermediate host.

1. **Adult** *E. multilocularis*.

2. **Ova** containing hexacanth embryo. The embryo or the onchosphère may develop into an alveolar hydatid cyst if ingested by an intermediate host.

3. **Humans**, presented as a dead end in the life cycle of the parasite. **Anthropozoonosis** is a subdivision of parazoonosis (Nelson, 1960).

4. **Alveolar hydatid cyst (AHC).** The larval stage of the parasite develops only in the intermediate host's tissues (liver, lung and peritoneum).
Echinococcus multilocularis
Leuckart 1863

DEFINITIVE HOST
A

LIFE CYCLE

INTERMEDIATE HOST
B

AHC

OVA

MAN

1

2

3

4
voles, squirrels, lemmings, shrews, and mice). The liberated hexacanth embryo passively migrates through the intestine to the viscera, mostly the liver, where they establish and proceed to grow. The larvae grow locally by exogenous budding. Metastasis to distant organs may occur due to the release of germinal cells into the circulation, infiltrating host tissue like a metastasizing tumor (Rausch 1954; Rausch and Schiller 1956; Obbayashi et al. 1971; Ali-Khan 1974; Wilson and Rausch 1980). The proliferating AHC is composed of several hundred small cysts, or brood capsules, each filled with hydatid fluid containing protoscolices. When the infected viscera is eaten by a definitive host, each protoscolex is capable of developing into a mature adult tapeworm thus completing the life cycle of the parasite.


The immunopathology of AHD is very much under investigated. Essentially chronic AHD is accompanied by hypergammaglobulinemia (Ali-Khan and Siboo, 1984) depressed cell mediated immunity (Ali-Khan, 1978a), immune complex I.C. mediated tissue injury and reticuloendothelial system RES dysfunction (Ali-Khan and Siboo, 1981). Tissue obliteration and mechanical obstruction by the proliferating larval cyst mass (LCM) (Cameron, 1960; Rausch, 1954) and renal failure (Aikat et al. 1978; Ozeretskovskaya et al. 1979). The lesion caused by the AHC in the liver, which is the primary site of infection, is characterized by a central zone of necrosis, cavitation and a peripheral zone of dense fibrous
tissue which is infiltrated by neutrophils, lymphocytes, plasma cells, histiocytes, giant cells and in some cases eosinophils (Rausch 1954; Rausch and Schiller 1956; Gamble et al. 1979; Ali-Khan et al. 1983; Treves and Ali-Khan 1984 a,b).

Recent interest in experimental alveolar hydatidosis originated with Lubinsky's work (1960a,b) which demonstrated that AHC infection can be maintained indefinitely in laboratory rodents through periodic transplantation of protoscolices or cysts. Since then a number of studies have clearly demonstrated a wide range of host susceptibility, ranging from completely refractory to hypersusceptibility, to AHC infection (Rausch and Schiller 1956; Ohbayashi et al. 1971; Ali-Khan 1974). Briefly, cotton rats, voles, and inbred strains of mice can be infected with eggs of E. multilocularis or intraperitoneal transplantation of fragments of AHC (cysts, protoscolices, or germinal layers). Each of these agents has the potential to develop into a solid tumor-like mass (Rausch 1954; Rau and Tanner 1972; Wilson and Rausch 1980).

Histologically the AHC in rodents appears as a honey comb consisting of small cysts and brood capsules intermeshed with the host's vascularized fibrous tissues and inflammatory cells (Rausch 1954; Rausch and Schiller 1956; Yamashita et al. 1958) The AHC grows either by exogenous budding of cysts, the extension processes from the germinal layer or the differentiation of germinal cells into cysts. Two forms of AHC - the progressive and restrictive types - have been recognized in various experimental hosts. The progressive AHC (contains brood capsules and
scolices) debilitates and kills the host (voles, cotton rats, and a number of inbred strains of mice) in 8 to 12 weeks. In contrast the restrictive AHC (with or without brood capsules or protoscolices) grows relatively slowly and takes longer to debilitate the host (humans, monkeys and a number of inbred strains of mice).

Rausch (1954), Rausch and Schiller (1956) and Yamashita et al. (1958) have described in detail the histogenesis of LCM and a qualitative description of concomitant inflammatory infiltrates. The progressing LCM were intact (i.e. devoid of central necrosis) and had relatively fewer inflammatory cells. Of these, neutrophils and macrophages constituted the dominant cellular infiltrates throughout the course of infection and eosinophils were rarely observed. In contrast, the restrictive LCM was characterized by central necrosis and an intense infiltration by inflammatory cells: eosinophils, lymphocytes, plasma cells, neutrophils and histiocytes were found in elevated numbers. In the above studies, no specific inflammatory cell was associated with cystolysis. The role of these cells in restricting the growth of the LCM remains to be delineated.

Baron and Tanner (1977), however, reported that combined thymectomy and antilymphocyte serum treatment increased metastatic dissemination and significantly increased the LCM weight. Cells of the monocyte/macrophage series have also been shown to suppress and abrogate the LCM in cotton rats previously sensitized with BCG (Reuben et al. 1978). This conclusion was based on the number of peritoneal cells in LCM-infected and BCG
challenged cotton rats but not on the number of cells in the LCM. Based on in vitro studies, two other host effector mechanisms were reported to lyse protoscolices isolated from LCM: macrophage-specific antibody-dependent (Rau and Tanner 1976) and complement (C) mediated lysis through the classical pathway of C activation (Kassis and Tanner 1976). In contrast, Rickard et. al. (1977) were unable to detect host antibody on the surface of protoscolices and found that protoscolices were lysed by the alternate pathway of C activation. Since protoscolices remain protected within brood capsules, and host protein has not yet been detected in brood capsules, it appears that C-mediated lysis of protoscolices in vitro has little or no significance in the in vivo situation. Furthermore, cysts, not protoscolices, are the invasive component of the LCM.

Alveolar hydatid disease, in the final analysis, is a chronic granulomatous disease. An intense and recurrent inflammation characterizes the course of AHC-infection in both humans and experimental host and a large proportion of the inflammatory cells infiltrate the stroma of the parasite biomass and the surrounding tissue. Despite this the AHC proliferates and survives until the host succumbs to the infection. Increasing experimental evidence indicates that the prolonged survival and uncontrolled growth of AHC may be due to compromised immunological functions in the host (Rausch and Schiller 1956; Ohbayashi et. al. 1971; Ali-Khan 1974). However, neither the AHC-elaborated pathogenetic factors have been identified nor their biological role in the pathogenesis of AHD is known.
Recently Ali-Khan et al. (1982) described amyloid induced obliterative changes in kidney glomeruli and spleen follicles of C57BL/6J mice infected 3-4 months earlier with the parasite. This finding was confirmed independently by Mettler et al. (1982) in Germany. They have described several amyloid deposits in the kidney interstitium, spleen follicles, pancreas and blood vessel walls of the gerbil (Meriones unguiculatus) 2-4 months post-infection. However, no information is available in the literature regarding the tissue distribution, time sequence of appearance and chemical composition of hydatid induced amyloid fibrils.

c. Treatment and Control.

There is no effective treatment for AHD. Surgical resection of the primary or secondary hepatic lesions in the early stages of the disease accompanied by mebendazole therapy is promising. A number of clinical trials using mebendazole in AHD-patients have proven efficacious (Bekhti et al. 1977; Kern et al. 1979; Wilson and Rausch 1980; Schantz et al. 1983). The treatment induced regression in the size of the lesion, a decline in the indirect hemagglutination titer to parasite antigens and prolonged the survival rate of the treated patients. The alternative approach to control the spread of AHD would be (1) to control the definitive host population, by the elimination of stray cats, dogs and wild carnivores from farm areas. (2) Continuous monitoring of the intermediate host population and the elimination of infected rodents, and (3) to better understand the
biology and pathogenesis of the disease in order to provide a mechanism to control the growth and proliferation of the AHC.

2. CLASSIFICATION AND PATHOGENESIS OF AMYLOIDOSIS.

Amyloidosis is a mesenchymal disease that associates itself with a variety of clinical disorders. It is characterized by the extracellular deposition of a fibrous protein, called amyloid, in a variety of tissues and organs of the body. The pathogenesis of amyloidosis varies among individuals depending on the site of deposition and the degree of disruption to the normal tissue architecture of the host. It can be of no clinical significance or it may lead to severe pathophysiologic changes resulting in the death of the affected individual. Although amyloidosis can appear by itself (idiopathic), its appearance is often preceded by chronic or long standing inflammation induced by a variety of agents (microbial, chemical or parasitic) (Gellhorn et al. 1946; Janigan and Druet 1966; Glenner 1980; Cohen 1982). Amyloid proteins differ in their chemical, physical and immunologic characteristics. Following the recent classification and nomenclature adopted at the proceedings of the Third International Symposium on Amyloidosis in 1979, amyloid fibril proteins have been divided into six major groups according to their chemical composition, site of deposition and underlying disease. These proteins are:

(1) Immunoglobulin (Ig) light chain proteins-derived (AL) amyloid (primary amyloidosis) associated with multiple
myeloma.

(2) Protein AA (secondary amyloidosis) associated with chronic inflammation, rheumatoid arthritis and familial Mediterranean fever.

(3) Pre-albumin-derived (AF) amyloid (Familial AF) associated with familial polyneuropathy.

(4) Endocrine tissue-related (AE) amyloid occurring in medullary carcinoma of the thyroid and the pancreas.

(5) Cutaneous or dermal (AD) amyloid occurring in tongue, skin etc.

(6) Pre-albumin-derived (AS) amyloid associated with senile dementia or Alzheimer's disease.

Amyloid proteins, regardless of their chemical nature, (a) stain metachromatically with crystal violet, (b) exhibit Congo red birefringence under polarized light (Ladewig, 1945), (c) stain with thioflavin T, toluidine blue and iodine (Cooper 1974), (d) exhibit tryptophan staining (Cooper 1969, 1974), (e) have a unique fibrillar appearance by electron microscopy (Ruinen et al. 1968), (f) show a characteristic x-ray diffraction pattern (Cohen and Calkins 1959; Cohen 1968), and (g) structurally, have an anti-parallel B-pleated sheet configuration (Hanes and Glenner 1968) (see figure 0.2).

a. Amyloid formation.

Two phases of development, a primary and secondary phase, characterize amyloid induction and deposition. Each phase is characterized by a considerable number of functional as well as
**Figure 0.2**

The meridional B-pleated sheet configuration of amyloid protein as defined by X-ray and infrared analysis showing the intramolecular antiparallel (A), intermolecular antiparallel (B) and intermolecular parallel (C) arrangement of protein molecules. The white arrow at center indicates the fibril axis and the black arrows indicate the direction of the protein from the amino terminal (N) to the carboxyl terminal (C*) amino acid. The distance between each protein molecule is 0.475 nm. All B-pleated sheet fibrils are congo red positive (birefringent) (Glenner et al, 1974).
B--PLEATED SHEET CONFIGURATION.
morphologic features. The histopathological changes during this biphasic development have been well described by several authors (Teilum 1954, 1956, 1964a, Franklin and Zucker Franklin 1972, Hardt and Ranlov 1976).

1. The primary phase. The duration of the primary phase, known as the preamyloidotic phase, varies among different species of animals. It depends upon the nature of the amyloidogen and the genetic factors of the host (Wohlgethan and Cathcart 1980). During this stage, large numbers of cells with large pale nuclei containing 1 or 2 nucleoli and a pyroninophilic cytoplasm appear in the perifollicular areas of the red pulp in the spleen, the endothelium of small blood vessels or sinusoids of the liver and other organs, and in close association with the mesangial cells of the glomeruli in the kidney (Teilum 1964a, b, 1968; De Brito et al. 1975; Watanabe et al. 1977, Chopra et al. 1984). The pathologic profile of this phase is complicated by lymphoid depletion, reflected by lymphopenia and involution of the white pulp in the spleen (Jaffe, 1926, Teilum 1964a, b, 1968; Franklin and Zucker Franklin 1972). It has been postulated that the induction period in amyloid formation is determined by the degree of lymphoid depletion from the spleen and lymph nodes. Druet and Janigan (1966) have demonstrated an inverse relationship between these two features. The shorter the induction period, as determined by the amount of antigenic stimulation, the more lymphoid depletion is observed and the heavier the pyroninophilia. In casein treated preamyloidotic mice the
pyronophilic spleen cells were identified to be mainly cells of the reticuloendothelial system (RES); plasma cells and B-lymphocytes constituted only a small percentage of these cells (Hardt and Ranlov 1972). However, Hardt and Claesson (1972) reported a significant increase in the number of T-bearing cells (T-cells) during this stage. The numbers were twice the normal value. Towards the end of the primary phase and before amyloid deposition, the splenic red pulp becomes filled with cellular debris including nuclear and cytoplasmic fragments as a result of cellular destruction. Pyknosis is perhaps the most important characteristic feature of the final stages of the primary phase. In addition a doubling in the normal rate of lymphoid cell decay is observed in the spleens and thymus of preamyloidotic mice (Claesson and Hardt 1972). However, the main cell type affected in this process is the T-lymphocytes (Claeson and Hardt 1972; Hardt and Claesson 1972). The restoration of T-cell function by the administration of thymosin has been shown to reduce the incidence of amyloidosis and significantly lengthen the induction period (Scheinberg et al. 1976b; Salvin and Neta 1983), thus emphasizing the role of T-cells in the pathogenesis of amyloidosis. In general, an increase in the rate of T-cell depletion (fragmentation) and histiocytosis in the spleen and thymus characterizes the final stages of the primary phase.

2. The secondary phase. The secondary phase, the amyloidotic phase, is characterized by the appearance of amyloid deposits. Amyloid deposition is initiated by the formation of an amyloid
accelerating or enhancing factor (AEF) (Janigan 1968; Ranlov and Wanstrup 1968; Axelrad et al. 1982; Kisilevsky and Boudreau 1983). Neither the precise site of origin nor the exact role of AEF and its mechanism of action is known. The initial stages of the secondary phase are characterized by an increase in the number of periodic acid Schiff (PAS)-positive RES cells in the perifollicular areas and a continued decrease in the T-cell population. Amyloid deposits appear first in the perifollicular areas in the red pulp as scattered isolated strands of amyloid. The deposits increase in thickness, join and form a ring of amyloid at the periphery and finally occupy the whole perifollicular area. The amyloid rings increase in thickness, expand into the red pulp and join other rings forming a continuous sheath of amyloid replacing more than 90% of the splenic tissue (Teilum 1956, 1968; Christensen and Hjort 1959; Ranlov 1976; Franklin and Zucker Franklin 1972).

b. Amyloid fibrillar structure.

The major constituent of amyloid fibrils in primary amyloidosis (plasma cell dyscrasia) is incomplete segments of the variable region of Kappa (k) or lambda (\(\lambda\)) immunoglobulin light chains and occasionally the heavy chains. The molecular weight (MW) of AL-amyloid is in the range of 18-22 K daltons (Glenner et al. 1969; Willerson et al. 1969; Pras et al. 1971; Glenner et al. 1972, 1974; Schinberg and Cathcart 1978; Westermark et al. 1981; Natvig et al. 1981). In secondary amyloidosis, which is associated with certain chronic inflammatory disorders,
protein AA constitutes more than 95% of the fibrillar component of the amyloid (Shirahama et al. 1967 a, b; Pras et al. 1968; Glenner et al. 1969; Harada et al. 1971; Westermark 1982). The MW of AA protein is approximately 8.5 K daltons. It has a relatively constant size and consists of 76 amino acid residues (Eriksen et al. 1976). The amino acid sequence is unique. It neither shows amino acid homology nor cross-reacts antigenically with any recognized AL-type of amyloid (Glenner et al. 1971; Benditt and Eriksen 1972; Skinner et al. 1974; Eriksen et al. 1976; Westermark 1982). The precursor of protein AA is believed to be a serum apolipoprotein of the high-density lipoprotein fraction called SAA (MW 160 K daltons). Experimental evidence shows that polypeptides similar to AA protein can be generated in vitro by enzymatic degradation of the parent SAA (Lavie et al. 1978; Skogen et al. 1980; Glenner 1980; Silverman et al. 1982). Furthermore, Hoffman et al. (1984) have recently shown an amino acid sequence homology between apo-SAA and protein AA fibrils, thus emphasizing the link between SAA and AA-protein.

Individual amyloid fibrils, regardless of their chemical nature, present a very characteristic appearance in ultrathin sections (see Fig. 1.8). Ranlov and Wanstrop 1968; Pras et al. 1968; Glenner et al. 1969; Duarte et al. 1978). Although they vary in length, they invariably measure 12 nm in diameter. The fibrils are oriented at random, but occasionally they are seen at right angles or parallel to each other and adjacent to cell membranes. They do not bend or branch and are always found in close association with pentagonally structured units of 23 K
Dalton size. These structures are called amyloid P-component (AP) (Bladen et. al. 1966; Cathcart et. al. 1967). The exact role of AP in the pathogenesis of amyloidosis is not known. The association of AP with the fibrils has been shown to be calcium dependent (Pepys et. al. 1977; Thompson and Enfield 1978). Amyloid P-component is a glycoprotein that constitutes 14-21% of the isolated amyloid fibrils (Cathcart et. al. 1971; Westermark et. al. 1975; Skinner et. al. 1983). Ultrastructural and immunohistochemical studies have shown that AP is morphologically similar and cross reacts with a normal plasma glycoprotein constituent called serum AP (SAP). No difference has yet been found in the properties of the two glycoproteins (Westermark et. al. 1975; Skinner et. al. 1983). Furthermore, Axelrad et. al. (1982), using the immunodiffusion test, have shown that anti serum against SAP cross reacts with crude preparations of AEF, indicating that AP is antigenically similar to AEF. However, upon further purification the SAP activity was claimed to dissociate from the purified AEF.

c. Site of synthesis of amyloid fibrils.

The precise mechanisms involved in the synthesis of amyloid fibrils from the soluble precursor subunits and its initial site of assembly are not known. The determination of the exact site of synthesis of amyloid fibrils has been a major problem aggravated by the state of the cells found in close association with amyloid deposits. The cells are often necrotic and damaged (Gueft and Ghidoni 1963; Sorenson 1964; Cohen 1965; Ranlov and
Wanstrup 1967). However, several authors have suggested, on the basis of experimental evidence obtained through ultrastructural and immunohistochemical studies, that amyloid formation may occur by one of 3 mechanisms: (1) excretion from the cell of origin as a soluble substance (preamyloid) which precipitates in the extracellular spaces in the form of amyloid fibrils, (2) premature intracytoplasmic assembly, or (3) a shift from extracellular to intracellular amyloid synthesis (Sorenson et al. 1964, 1966; Cohen et al. 1965; Ranlov and Wanstrup 1967; Zucker Franklin and Franklin 1970; Franklin and Zucker Franklin 1972). The fact that morphologically identical fibrils to amyloid have been described in normal epithelial cells from healthy individuals (Fawcett, 1966; Buckley and Porter 1967) and the demonstration of an intracellular amyloid precursor substance in the peripheral blood monocytes of amyloidotic patients suggests that amyloid formation may be initiated intracellularly and then deposited extracellularly for uptake by phagocytic cells (Wegelius 1974; Hardt and Ranlov 1976). Alternatively, Kisilevsky (1983) has proposed that SAA to AA conversion may occur extracellularly at precise anatomic locations as a result of aberrations in local RES cells. However, the precise nature of aberrations of these cells are unknown.

Glenner et al. (1981) have implicated defective intralysosomal proteolytic activity of the cerebral endothelial cells in the pathogenesis of amyloid plaques in Alzheimer's disease. Substances that have physical characteristics of amyloid have been created in vitro from insulin, and Bence Jones
light chains by digestion with pepsin, trypsin, kidney lysosomal extracts or by heating in acid (Glenner et al. 1974). It is therefore logical to assume that amyloidogenic AA-polypeptides are generated locally at precise loci and involve enzymatic degradation of precursor protein by RES cells. However, identification of pathogenic factors which induce the conversion of normal or abnormal host serum constituents into fibrillar amyloid deposits still remains an urgent and challenging problem in amyloidosis.

d. Cell of origin.

Several investigators studying splenic, liver and kidney tissues during the secondary phase of amyloid induction provided experimental evidence to show that the cells of the RES are involved in amyloid fibril formation (Heefner and Sorenson 1962; Laufer and Tal 1967). Either a soluble precursor or amyloid deposits were identified in the cytoplasmic matrix or in close association with the plasma membrane of these cells (Ben-Ishay and Zlotnik 1968; Zucker Franklin and Franklin 1970; Zucker Franklin 1974). Further evidence was provided when intracellular or intralysosomal amyloid fibrils were found in the RES cells of the spleen, bone marrow (Ben-Ishay and Zlotnik 1968; Shirahama and Cohen, 1975) and the senile plaques of the central nervous system of patients with senile amyloidosis (Terry 1963, 1964). Cells other than the RES have also been incriminated in the formation of amyloid fibrils. Sorenson and Shimamura (1964) reported that the first fibrillar deposits in the kidneys occur
in the mesangium of the glomerulus. In humans the mesangial cell matrix and basement membrane were shown to be the earliest sites of amyloid deposition in renal amyloidosis (Suzuki 1963). These observations were later confirmed by Duarte et al. (1978). They have shown that amyloid deposits occur in close association with functionally impaired mesangial cells. It was suggested that the amyloid precursor material is secreted into the matrix by mesangial cells. Shibolet et al. (1967), on the other hand, suggested that kidney fibroblasts located between the capillary and tubular membranes in leishmaniasis infected hamsters are involved in the deposition of amyloid. Teilum (1956, 1964 a, b) and Caesar (1960) incriminated plasma cells in the synthesis process. Zucker Franklin and Franklin (1970) have demonstrated the presence of amyloid fibrils in the cytoplasm of monocytoïd and plasma cells that were adjacent to or surrounded extensive extracellular amyloid deposits.

3. TRANSFER OF AMYLOIDOSIS.

While studying the functional aspects of the immune system in amyloidotic mice, Werdelin and Ranlov (1966) found that the transfer of viable spleen cells from amyloidotic C3H mice (casein induced) into syngeneic normal mice induced amyloid deposition in the spleens of the recipients within 3-5 days following antigenic stimulation with casein or nitrogen mustard. Several other investigators have successfully reproduced and elaborated on this important observation (Janigan and Druet 1968;
Ranlov 1968; Janigan et al. 1969; Hardt and Hellung-Larsen 1972). The finding of a transfer factor in amyloidotic animals and humans is perhaps the most important discovery in the history of amyloidosis. Recent studies on the "transfer factor" or "AEF" have firmly established its role in the induction of amyloid deposition. This factor appears in the tissues (mainly spleens) of preamyloidotic animals 24-48 hours preceding the appearance of amyloid fibrils. (Axelrad et al. 1982; Cohen et al. 1983; Kisilevsky and Boudreau 1983).

a. Tissue source of the transfer factor.

The spleen is considered by several investigators as the best source of the transfer factor (Hardt 1968; Ranlov 1968; Janigan and Druet 1968; Axelrad et al. 1982). The transfer has been performed successfully with either of the following: (1) whole spleen grafts (Hardt 1968) (2) varying numbers of viable spleen cells (25 x 10⁶ to 200 x 10⁶) in physiological medium (Werderlin and Ranlov 1966; Hardt and Ranlov 1976; Cathcart et al. 1972) or (3) non viable spleen cells (Ranlov 1967). Furthermore, homogenized suspensions of livers or lungs as well as sera obtained from amyloidotic animals were also found to possess the transfer factor although to a much lesser extent than spleens (Willerson et al. 1969; Janigan and Druet 1968). On the other hand, thymus and lymph node cell suspensions from amyloidotic animals failed to induce amyloid deposition in syngeneic mice (Hardt 1971; Hardt and Ranlov 1976).
b. **Cellular source of the transfer factor.**

Several attempts have been carried out in order to transfer amyloidosis with one or more homogeneous cell populations (Clerici et al. 1969). Fifty or 100 x 10^6 lymphocytes from the thymus, lymph node, bone marrow, peritoneal cavity and peripheral blood, 25 x 10^6 peritoneal macrophages or 100 x 10^6 bone marrow cells plus thymocytes (1:1 ratio) when transferred to syngeneic mice failed to induce amyloid deposition. The only combination of cells that induced amyloid deposition comparable to the control group were spleen cells devoid of T-lymphocytes (spleen cells of a nu/nu-C3H, nude mice). The recipients were normal C3H mice (Hardt and Claesson 1974). However, when the recipients were nude mice, the transfer failed to induce amyloid deposition. No explanation has been given for this important observation.

c. **Transfer with subcellular fractions.**

Ranlov (1967b), found that damaged or nonviable spleen cells do transfer amyloidosis at the same rate as viable ones. Several attempts were carried out in order to localize the activity of the transfer factor in a subcellular fraction. Ranlov (1967b) separated spleen cell homogenates into 2 fractions. The first fraction consisted of DNA, DNA-linked proteins and histones while the second fraction consisted of RNA, mitochondria and microsomes. The inoculum was administered i.v. and was equivalent to 100 x 10^6 cells. Only the nuclear fraction induced amyloid formation within 2-4 days. However, when the
first fraction was incubated with DNAase, the activity was not abolished. On the other hand, Willerson et al. (1969) in a similar experiment found the mitochondrial and microsomal fractions most effective. They found an insignificant amount of activity in the nuclear fraction. However, in both of these experiments the purity of each fraction was not assessed before administration into animals. In a more detailed and controlled experiment, using 3 different methods for the separation of the subcellular fractions, Hardt and Hellung-Larsen (1972) found that the activity can be transferred with nuclear elements and not with RNA or cytoplasmic components. Unlike Ranlov's observations, the activity of the nuclear fraction was completely abolished when treated with DNAase. The purity of each fraction was assessed by electron microscopy and polyacrylamide gel electrophoresis (PAGE), and the nuclear fraction was pre-treated with diethylpyrocarbonate, which is a very potent RNAase-inhibitor. They have also shown that the transfer factor loses activity when incubated at 37°C for 2 hours or stored at 0°C or 20°C for 3 days. It is important to note that xenogenic transfer of amyloidosis across the species barrier was successfully achieved. Hardt and Ranlov (1976) and Janigan and Druet (1968) transferred amyloidosis from amyloidotic human spleens to C3H mice.

d. The **Amyloid Enhancing Factor** (AEF).

Successful transfer of amyloidosis was also achieved when spleen cells from preamyloidotic animals were transferred to
syngeneic or allogeneic animals and followed with the same antigenic stimulation as the donor mice (Hardt and Ranlov 1968; Janigan and Druet 1968). The depletion of the macrophage and B-cell populations from the donor spleen cells did not alter the activity of the enhancing factor (Hardt et al. 1975). Similar to the transfer factor, the main source of the amyloid enhancing factor (AEF) is the spleen. However, the thymus, axillary lymph nodes and peritoneal cells were found to transfer amyloidosis though to a lesser extent (Hardt 1971; Cathcart et al. 1973). In an attempt to localize the source of AEF at the subcellular level, Janigan (1968) and Jacob and Hilgenfeld (1972) injected cytoplasmic or nuclear fractions of spleen cell homogenates and lymph node homogenates into syngeneic or allogeneic mice. All the recipients, regardless of the fraction received, developed amyloidosis following an azocasein injection, indicating that AEF activity was present in all the subcellular fractions of the cells. However, one should be cautious in the interpretation of data based on experiments dealing with crude preparations of an unknown substance. For instance, it is not possible to determine with sufficient accuracy the exact amount of transfer or enhancing factor(s) either from intact spleen cells or cell extract preparations. Until recently, the exact nature of AEF was not known. In an elegantly planned and controlled set of experiments Drs. Michael Axelrad and Robert Kisilevsky of Queen's University have shown that AEF is a high molecular weight glycoprotein. They found AEF activity in all the subcellular fractions (microsomes, ribosomes and membranes devoid of
ribozymes) except the 100,000 x g spleen cell extract supernatant (Axelrad et. al. 1982). They have also shown that AEF is not the amyloid P-component, its serum counterpart SAP or interleukin-1 (Kisilevsky et. al. 1979; Axelrad et. al. 1982; Kisilevsky and Boudreau 1983). AEF appears just 24-48 hours prior to amyloid deposition in the host spleens or livers (Axelrad et. al. 1982; Kisilevsky and Boudreau 1983), and is resistant to enzymatic degradation with DNAase, RNAase, lipase and amylase (Axelrad and Kisilevsky 1980). However, considerable loss of activity was observed when AEF was treated with pronase and sodium periodate (100 mM) and a complete loss of AEF-activity occurred when it was incubated at 95°C or 100°C for 1 hour (Axelrad and Kisilevsky 1980).
4. OBJECTIVES OF THIS STUDY:

This study was undertaken to investigate the following:

(1) Amyloidogenic potential of alveolar hydatid cyst in mouse strains of varying susceptibility to the parasite,

(2) Effect of inoculum size on amyloid deposition (number of cysts in the infective dose) on the onset, incidence and amount of amyloid deposition in target organs,

(3) Effect of the site of infection on the onset, incidence and amount of amyloid deposition in target organs,

(4) Pre- and post-amyloidotic pathological changes in the splenic and hepatic tissues of the host,

(5) Ultramicroscopic, histochemical and immunohistochemical characterization of the amyloid,

(6) Isolation, purification and amino acid analysis of the amyloid,

(7) Identification and functional characterization of the parasite-derived factors which may be involved in the pathogenesis of alveolar hydatid cyst-induced amyloidosis,

(8) Isolation and functional characterization of an amyloid enhancing-like factor from the spleens of alveolar hydatid cyst-infected mice.
The experimental results in this thesis are presented in 4 chapters. Each chapter contains introduction, materials and methods, results and discussion.
CHAPTER ONE.

CHRONIC ALVEOLAR HYDATIDOSIS AND SECONDARY AMYLOIDOSIS:
PATHOLOGICAL ASPECTS OF THE DISEASE IN FOUR STRAINS OF MICE.
1. INTRODUCTION.

Secondary amyloidosis is frequently the major complication of several chronic inflammatory diseases. The incidence of the AA type of amyloidosis is relatively high among patients with granulomatous diseases of known aetiology such as tuberculosis, leprosy and osteomyelitis (Cohen, 1967). In contrast the association between parasitic infections and amyloidosis is rather sketchy. It was first demonstrated in *Leishmania* infected hamsters by Gellhorn et. al. (1946) and subsequently in human leishmaniasis (Hinglais and Montera, 1964). Metazoan parasite-induced amyloidosis has also been reported in rodent filariasis (Crowell and Votava, 1975), human schistosomiasis (Andrade and Rocha, 1979; Barsoum et. al., 1979) and rodent and human alveolar hydatidosis (Ozeretskovskaya personal communication; Ali-Khan et. al., 1982, 1983; Mettler et. al., 1982). High incidence of amyloidosis was recorded in patients with the metastatic form of alveolar hydatidosis.

Despite intense investigations during the last two decades using the casein-mouse experimental model, the pathogenesis of secondary amyloidosis still remains an enigma. We have recently described an alveolar hydatid cyst-mouse model for the study of secondary amyloidosis (Ali-Khan et. al., 1983). The purpose of the work reported in this chapter is (a) to compare the amyloidogenic potential of the parasite in different host
strains, (b) to examine the pre-, and post-amyloidotic tissue alterations in target organs, (c) to determine the time sequence and organ distribution of amyloid deposition and, finally (d) to determine the effect of the inoculum size and site of infection to the induction of amyloidosis. The results further establish the value of this novel experimental model to explore the pathogenesis of secondary amyloidosis as a sequela of chronic infectious diseases.

2. MATERIALS AND METHODS

a. Mice.

Male mice (A/JAX H-2a; C57BL/6J, H-2b; Balb/c, H-2d and CBA/J, H-2k) between 6 to 8 weeks of age and weighing approximately 20g were purchased from Jackson Laboratories, Bar Harbor, Maine, U.S.A. Forty mice of each of the four strains were inoculated intraperitoneally with 50 ± 10 cysts of *Echinococcus multilocularis*. This dose was chosen because a number of host responses were characterized using this inoculum (Ali-Khan et al., 1983).

b. The Parasite.

The historical background of the strain of *E. multilocularis* used in this study and its maintenance in the laboratory have been published elsewhere (Ali-Khan 1978a). Briefly, since 1978 the parasite has been maintained in C57BL/6J mice by i.p. or s.c. transfer of 50 to 100 cysts from hydatid mice infected 12 to 16 weeks previously. The parasite was always
passaged at least once through the mouse strain being used in the experiments; this precaution was taken in order to eliminate any host allograft responsiveness towards the donor strain.

c. **Inoculum size and route of infection.**

To determine the effect of the size and site of the infective inoculum on amyloid induction, forty C57BL/6J mice were divided into four groups of 10 mice each. Mice in each group were inoculated intraperitoneally (i.p.) with 5, 50 or 250 cysts or subcutaneously (s.c.) with 50 cysts by the method previously described (Ali-Khan, 1978a). Essentially, the LCM from i.p. or s.c. infections after 12 weeks were excised at necropsy. The alveolar hydatid cysts (AHC) were freed from the LCM by mechanical disruption. They were collected by sedimentation in a 1 liter graduated cylinder and washed extensively in Hanks balanced salt solution, pH 7.2. The isolated AHC were placed in a petri dish and the inoculum was picked up by means of a syringe with an 18 gauge needle, with the aid of a dissecting microscope.

d. **Preparation of tissue for histologic examination by light and electron microscopy.**

Four to eight mice from each group were sacrificed at 2, 4, 6, 8, and 12 weeks post-infection (p.i.). The intraperitoneal larval cyst masses (LCM) were collected and weighed. Pieces of kidney, liver spleen, stomach, large and small intestine, tongue, urinary bladder, brain, heart, pancreas, lungs, femur and diaphragm were fixed in 10% buffered formal saline immediately
after the mice were sacrificed. Paraffin sections 4, 7, and 12 μm thick were prepared and stained with Congo red stain following the alkaline Congo red method of Puchtler et al. (1962). Five to ten uninfected mice of each mouse strain served as controls. These mice were approximately five months old at the time of tissue sampling. All Congo red-stained sections were examined under polarized light using a Dialux 20 (Wild MPS45) light microscope. Amyloid deposits were identified by their dense green birefringence.

For electron microscopy, spleen, liver and kidney samples obtained from C57BL/6J mice at 8 and 12 weeks p.i. (2 x 5 mm in size) were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 6-12 hours. They were washed in phosphate buffer, dehydrated in ascending grades of alcohol and embedded in Epon. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope.

e. Quantitative morphology.

Congo red stained sections of spleens, livers and kidneys were used to assess the cross sectioned surface areas occupied by amyloid deposits. The morphometric method of Weibel et al. (1966) was followed with slight modification. The slide projector was substituted with a microprojector (Leitz Microprroman, Wild Leitz Canada Ltd.). The projected field was reflected at a 90° angle using a mirror and was superimposed with a double lattice test-system. The location of each surface area
covered with amyloid (showing green birefringence with polarized light microscopy) was traced on the test sheet. The percentage of amyloid deposits in a cross sectional area of a specific tissue was determined using the following formula:

\[
\frac{\text{surface area of the total number of squares covered with amyloid in a given field}}{\text{surface area of the total number of squares in that field}} \times 100
\]

A minimum of 25 fields were recorded per tissue for each specific time post-infection. Estimates of kidney involvement by amyloid is based on the amount of glomerular, tubular and interstitial deposits and is graded on a scale of (+) to (+++). Amyloid deposits covering an area ranging between 1-25% are given a grade of (+), 25-75% is given (++) and if more than 75% it is graded (+++).

f. Statistical analysis.

The Wilcoxon, Mann-Whitney "U" test and the t-distribution test were used to analyze the data of all experiments in order to compare the mean of treated and untreated groups of mice. The data was considered significant if the "Ho" or "t" values were above the 5% level (Steel and Torrie, 1960). The "Q" test was also used in order to eliminate extraneous values which failed to pass the screening test (Dean and Dixon, 1951). Questionable data was rejected with 90% confidence when the calculated "Q" value exceeded the tabulated one.
3. RESULTS

a. Parasite biomass and host-strain susceptibility to amyloidosis.

Congo red stained paraffin sections of various soft organs were examined under polarized light microscopy to detect the onset, progression and extent of tissue involvement by amyloid. Spleens were the first organ to become positive for amyloid and none of the hydatid mouse became positive until 6 weeks p.i. The intensity of the greenish birefringence was much reduced when 4 μm instead of 7 or 12 μm thick sections were examined. The data presented here are based on 7 μm thick sections.

Table 1.1 summarizes the mean LCM weights and Fig. 1.1 illustrates the organ distribution of amyloid at corresponding time-intervals. All four hydatid-mouse strains (Balb/c, C57BL/6J, CBA/J and A/JAX) were susceptible to amyloidosis to varying degrees. They showed essentially similar amyloid-mediated tissue lesions initially. Subsequently as the infection progressed a discernable difference in the rate of amyloid deposition and pathomorphologic alterations were observed. With respect to amyloidogenesis, A/JAX strain was highly resistant, Balb/c and C57BL/6J strains were highly susceptible while CBA/J strain occupied an intermediate position between these two strains (Figs. 1.1 & 1.2). Growth of the LCM was relatively rapid in Balb/c mice. At 6 weeks p.i. the mean LCM weight was 1.46 g and all the mice were positive for amyloid.

Susceptibility of soft organs to amyloid deposition (spleen 100%, liver 100%, kidney 50%) was, however, variable (Fig. 1.1).
Table 1.1

Mean larval cyst mass weights (LCM) in grams at 2, 4, 6, 8 and 12 weeks p.i. from C57BL/6J, Balb/c, CBA, and A/JAX mice infected i.p. with 5, 50 or 250 cysts or s.c. with 50 cysts of *E. multilocularis*

<table>
<thead>
<tr>
<th>Weeks Postinfection</th>
<th>C57BL/6J</th>
<th>Balb/c</th>
<th>CBA/J</th>
<th>A/JAX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>2</td>
<td>0.17±</td>
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<td>0.12±</td>
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<td>1.0±</td>
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<td>0.792±</td>
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<td>12</td>
<td>4.8±</td>
<td>3.6±</td>
<td>6.1±</td>
<td>3.3±</td>
</tr>
</tbody>
</table>

1. Intraperitoneal infection with 50±5 cysts
2. Subcutaneous infection with 50±5 cysts
3. Intraperitoneal infection with 250±10 cysts
4. Intraperitoneal infection with 5 cysts
Figure 1.1

Incidence of splenic (solid bar), hepatic (open bar) and renal (striped bar) amyloidosis at various time intervals from C57BL/6J, Balb/c, CBA/J and A/Jax mice infected intraperitoneally (i.p.) with 50 cysts of E. multilocularis.
Figure 1.2

Measurement of splenic (open circles) and hepatic (closed circles) amyloid deposits at various time intervals from C57BL/6J (dotted line), Balb/c (solid line), CBA/J (broken line) and A/Jax (single circle) mice infected i.p. with 50 cysts of E. multilocularis.
C57BL/6J mice the threshold for amyloidogenesis with reference to the mean LCM weight was much lower. At 6 weeks p.i., 3 of 8 C57BL/6J mice spleens became positive for amyloid when the mean LCM weight in these mice was three times smaller than that of Balb/c strain. CBA/J and A/JAX mice did not become positive for amyloid until 8 and 12 weeks p.i. at which time their mean LCM weights were 1.34 and 5.4 g, respectively. As with the Balb/c and C57BL/6J susceptible strains, the first target organ for amyloid deposition in the CBA/J strain was the spleen; 3 of 8 spleens had amyloid deposits. At 12 weeks p.i., the mean LCM weight in Balb/c mice was 10.4 g which was approximately 2 times larger than those isolated from C57BL/6J, CBA/J and A/JAX hydatid-mice. The incidence of amyloidosis, however, in these four hydatid host-strains did not correspond to the load of the parasite biomass (Fig. 1.1, Table 1.1).

b. Effect of inoculum size and site of infection:

The mean LCM weights and the incidence of amyloid deposits in various organs during the course of infection are presented in Table 1.1 and Fig. 1.3. Neither increase or decrease in the size of intraperitoneal inoculum (250 or 5 instead of 50 cysts) nor the difference in the site of infection altered the induction period of amyloid. Mice in both groups were positive for amyloid at 6 weeks p.i. The mean LCM weights were similar in both groups of mice and comparable to that of 50 cyst intraperitoneally-infected C57BL/6J mice (Table 1.1).
Figure 1.3

Incidence of splenic (solid bar), hepatic (open bar) and renal (striped bar) amyloidosis at various time intervals from C57BL/6J mice infected subcutaneously (s.c.) with 50 cysts or i.p. with 5, 50 or 250 cysts of E. multilocularis.
% Amyloid positive

Weeks Post-infection

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.C.</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>I.P.</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>0</th>
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<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
c. **Histology.** Among the various organs and tissues examined, amyloid deposits were detected in the spleen, liver, kidney, stomach, small and large intestine, urinary bladder and pancreas. No deposits were found in the brain, heart, tongue, lungs, diaphragm, and bone marrow. In order to further characterize the incidence and degree of organ sensitivity to amyloid deposition and their possible relationship to the parasite-load, pathological changes and the sequence of amyloid deposition are described in spleens, livers, and kidneys from the four hydatid-mouse strains.

d. **Spleen.**

The hyperplastic changes and the pattern of amyloid deposition were similar in Balb/c, C57BL/6J, CBA/J and A/JAX spleens. The preamyloidotic alterations were characterized by perifollicular hypercellularity, expansion of red pulp, enlargement of follicles, a significant increase in the number of megakaryocytes in the red pulp and appearance of hyperplastic germinal centers. Monocytoid cells were the predominant infiltrates in the perifollicular sinuses. Amyloid fibrils first appeared as isolated strands in the perifollicular area. As infection progressed, the isolated strands coalesced, thickened and progressed towards the center of the follicle. Progressive hypocellularity of the red and white pulps and follicular atrophy marked the postamyloidotic alterations.

At 6 weeks p.i., Balb/c and C57/6J spleens showed preamyloidotic alterations as well as green birefringent
deposits. Amyloid-mediated alterations were more severe in Balb/c mice. The perifollicular areas were essentially noncellular and had a solid sheet of amyloid. Most of the radial sinuses were obliterated; those which were patent were devoid of cells. The germinal centers were partially atrophied and occasionally showed strands of amyloid. Endothelial cell nuclei of central arterioles were enlarged but the walls of the central arterioles were generally devoid of amyloid deposits. Only one Balb/c mouse spleen that had heavy amyloid deposits showed subendothelial deposits in the central arteriole. The red pulp was negative for amyloid. It was hypercellular and contained a moderate number of megakaryocytes and myeloid infiltrates. The latter had donut-shaped nuclei suggestive of immature granulocytes. In C57BL/6J mice most of the perifollicular amyloid deposits appeared as disoriented scattered fibrils or were fused to form a narrow ring. Occasional follicles had their radial sinuses obliterated by amyloid. CBA/J and A/JAX hydatid-mice spleens were hyperplastic but negative for amyloid (Fig. 1.1).

At 8 weeks p.i. all Balb/c and C57BL/6J hydatid-mice were positive for amyloid and showed extensive obliteration of the splenic follicles and red pulp (Figs. 1.1 and 1.2). Balb/c mice, however, harboured LCM 3 times larger than C57BL/6J mice (Table 1.1) and their (Balb/c) spleens contained abundant deposits (Fig. 1.2). Remnants of partially obliterated follicles appeared as small clusters of lymphocytes engulfed in an essentially noncellular sheet of eosinophilic material. Topographically this
was the result of fusion between amyloid deposits in the follicles and the red pulp. The latter became progressively hypolecullar but the frequency of myeloid foci increased in some relatively intact portions of the red pulp. The walls of most central arterioles were thickened and had heavy amyloid deposits. Their endothelial cell nuclei appeared dense and hyperchromic. None of the A/JAX mice, and only 3 of 8 CBA/J mice spleens, were positive for amyloid. Amyloid-induced alterations in the splenic tissue of CBA/J mice were comparable to those of C57BL/6J hydatid-mice at 6 weeks p.i. Microscopic changes in A/JAX and CBA/J spleens negative for amyloid were consistent with the preamyloidotic stage of the infection.

At 12 weeks p.i. extensive amyloid deposition had nearly completely obliterated the follicles and red pulp in C57BL/6J and Balb/c mice. Splenic architecture was radically altered and hardly recognizable. In C57BL/6J mice more than 90% of the follicles appeared as amorphous eosinophilic nodules (Fig. 1.4a). In Balb/c mice remnants of most follicles were still recognizable. In both host strains the red pulp was extensively devastated by amyloid; only small scattered foci of monocytoid and myeloid cells were found in the red pulp. The walls of most of the central arterioles were thickened, fibrotic and heavily infiltrated by amyloid and some had lost their endothelial lining. All CBA/J mice, at 12 weeks p.i., were positive for amyloid. The histologic alterations in the spleens were comparable to those of 12 weeks p.i. C57BL/6J mice; 2 of 8 CBA/J spleens had more amyloid deposits than the remaining six spleens.
Figure 1.4

Hydatid mouse spleen at 12 weeks post-infection (p.i.) Congo red stain, X 100.

(A) C57BL/6J mouse. Remnants of two splenic follicles and portions of the red pulp show obliteratorative changes by amyloid deposits. Note the thickened central arteriolar wall without endothelium and a megakaryocyte in the lower center. The perifollicular amyloid "ring" is devoid of cells.

(B) A/Jax mouse. Note two relatively intact splenic follicles with perifollicular amyloid deposits and clumps of amyloid in the red pulp.
Five of eight A/JAX hydatid-mice spleens became positive for amyloid at 12 weeks p.i. The perifollicular ring of amyloid was of variable thickness and the splenic alterations were histologically comparable to those of Balb/c mice at 6 weeks p.i. The follicles were morphologically intact (Fig. 1.4b). The red pulp was expanded, displayed an increased number of megakaryocytes and relatively small clumps of amyloid deposits which had not fused together to form an amorphous sheet. The cumulative sectional areas of spleens occupied by amyloid in Balb/c, C57BL/6J, CBA/J and A/JAX mice are given in Fig. 1.2

2. Liver.

At 6 weeks p.i., the periportal and centrilobular accumulation of inflammatory cells was several layers in thickness and prominent Kupffer cell nuclei were evident in all Balb/c hydatid mice (Fig. 1.5a). Affected portal tracts and sinusoids expanded; sinusoids were frequently packed with monocytes, lymphocytes and occasional plasmacytoid cells constituted the perivascular infiltrates (Fig. 1.5b). Hepatocytes adjacent to the outer margin of the inflammatory cells were foamy and their nuclei were either swollen or fragmented into chromatin granules. All Balb/c livers were positive for amyloid (Fig. 1.1). Amyloid deposits were confined to the walls of central veins and sinusoids. Perivascular deposits, if present, were found among the inflammatory cells.

Liver sections from 6 weeks p.i. C57BL/6J, CBA/J and A/JAX
Figure 1.5

Hydatid mouse liver at 6 and 12 weeks p.i. Congo red stain.

(A) Balb/c mouse at 6 weeks p.i. Note the prominent Kupffer cell nuclei, focal inflammation in the hepatic parenchyma and periportal and centrlobular accumulation of inflammatory cells X 100.

(B) High-power view of a Balb/c mouse liver at 6 weeks p.i. showing monocytoid cells and granulocytic infiltrates encroaching upon the hepatocyte. X 400.

(C) C57BL/6J mouse at 12 weeks p.i. Note birefringent deposits (polarized microscopy) involving sinusoids, hepatic veins and portal areas. Perivascular infiltrates are present at the outer margin of birefringent deposits. X 100

(D) Low-power view of a C57BL/6J mouse at 12 weeks p.i. showing intense perivascular and diffuse hepatic inflammation, inflammatory cells plugging sinusoidal lumina and thickening of the vessel walls. X 100
Mice appeared normal. However, occasionally in the former two host strains, sinusoids were packed with monocytoid cells and lymphocytes; a few of the sinusoids contained predominantly immature granulocytes. In addition, a few portal vessels in C57BL/6J mice showed amyloid deposits and perivascular clusters of granulocytes and monocytoid cells. None of the liver sections from CBA/J or A/JAX mice were positive for amyloid (Fig. 1.1).

At 8 weeks p.i. the perivascular cuff of inflammatory cells had enlarged in Balb/c mice and hepatic architecture was significantly altered. Large areas of hepatocytes were replaced by monocytoid cells and granulocytic infiltrates. An increased number of sinusoids were packed with granulocytes and monocytoid cells. Amyloid deposits were either perivascular or involved the vessel walls. In the former case, the amyloid deposits often formed a complete ring and inflammatory cells invariably separated the vessel wall from the amyloid ring. Most of the vessels which were positive for amyloid showed eosinophilic thickening and loss of endothelium. The lumina were either empty or plugged with inflammatory cells. At 8 weeks p.i. liver sections from 2 of 8 CBA/J−, and all C57BL/6J-hydatid mice were positive for amyloid. Histologically they were comparable, with some exceptions, to that of Balb/c mice at 6 weeks p.i. Firstly, portal and centrilobular vessel walls had segmented amyloid deposits. Rarely was the entire vessel wall involved. Secondly, sinusoidal walls were rarely positive for amyloid. Thirdly, perivascular and intrasinusoidal infiltrates were predominantly lymphocytes and monocytoid cells; focal accumulation of
granulocytes was rare. Liver sections from all A/JAX-hydatid mice were negative for amyloid.

At 12 weeks p.i. the architecture of the liver of both Balb/c and C57BL/6J hydatid mice was severely distorted. Hepatocellular disruption and partial or complete obliteration of portal areas by inflammatory cells or amyloid was a common finding (Figs. 1.5 c and d). However, quantitatively a larger area of the hepatic parenchyma of Balb/c mice was infiltrated by amyloid deposits (Fig. 1.2). Similarly they had heavier cellular infiltrates; mature and immature granulocytes were the predominant cells. Most of the vessels were severely disrupted. The endothelial layer had detached and the entire vessel wall was replaced by a solid ring of amyloid (Figs. 1.6 a and b). The vessel lumen often showed a plug of inflammatory cells and the space between the amyloid ring and endothelium was invariably infiltrated by monocytoid cells and granulocytes. Sinusoids, adjacent to central veins with heavy amyloid deposits, were plugged with amyloid. This had severely disrupted the liver trabeculae. As a rule the liver sinusoids under the capsule were more severely affected than those in the deeper liver parenchyma. A large proportion of liver cells were hyperchromic and their nuclei were either hyperchromic or of variable sizes.

Liver histology of CBA/J and A/JAX-hydatid mice at 12 weeks p.i. was comparable to that of C57BL/6J mice at 8 and 6 weeks p.i., respectively. Four of 8 A/JAX livers and all CBA/J livers were positive for amyloid (Fig. 1.1). Minimal tissue alterations, either by amyloid deposits or inflammatory cells
**Figure 1.6**

C57BL/6J hydatid mouse liver at 12 weeks p.i. High power view of a central vein and surrounding hepatic parenchyma with (A) and without (B) polarized light. Note the detached endothelium, monocytoid cells and neutrophilic infiltrates between the endothelium and the vessel wall with heavy birefringent deposits. The sinusoidal walls and lumina are also heavily infiltrated by amyloid. Congo red stain, X 400.
were noted in A/JAX mice (similar as Fig. 1.6 a and b). Figure 2. illustrates the extent of hepatic parenchyma obliterated by amyloid during the course of infection in the 4 hydatid-mouse strains.


Figure 1.7 illustrates the incidence of renal amyloidosis and Table 2 summarizes the degree of glomerular, tubular and interstitial involvement in the four hydatid-mouse strains at various time-intervals. Consistent differences were observed both in the severity of kidney lesions and the time of appearance of fibrillar deposits. The deposits were heavier and had a wider distribution in the kidneys of C57BL/6J and Balb/c than CBA/J or A/JAX strains. The former two host strains showed the earliest deposits at 6 weeks p.i.; amyloid fibrils involved both the glomerular capillaries and mesangium had a segmental distribution (Fig. 1.7a). As infection progressed, increasing numbers of hydatid-mice showed renal involvement. Glomerular deposits became widely distributed (Fig. 1.7b). Other areas of the kidney, especially the interstitium of the renal papilla, the basement membrane of the renal tubules in both the cortex and medulla and the intertubular capillary walls became increasingly involved with amyloid.

At 12 weeks p.i., 100% of C57BL/6J and 62% of Balb/c mice kidneys were positive for amyloid. About 50% of the C57BL/6J glomerular tufts appeared like a cauliflower and approximately two-thirds of their sectional areas were infiltrated by amyloid
Figure 1.7

C57BL/6J hydatid mouse kidneys showing segmented (A) and diffuse (B) distribution of birefringent deposits (polarized light) at 6 and 12 weeks p.i., respectively. The deposits involve the mesangium, glomerular capillaries and the interstitium. Congo red stain, X 400.
fibrils. The interstitium of the medulla was also infiltrated by amyloid (Table 1.2). The extent of kidney lesions in Balb/c mice was relatively less severe. Despite heavy amyloid deposits in the glomeruli, glomerular capillaries in Balb/c and C57BL/6J mice remained patent. Amyloid deposits were quite variable and mainly segmental in distribution in the kidney glomeruli of CBA/J and A/JAX mice. Interstitial deposits were infrequent (Table 1.2).

Exudation, necrosis of cellular infiltrates were never observed in any part of the kidney at any stage of infection. Renal casts, however, appeared more frequently in C57BL/6J and Balb/c mice between 8 and 12 weeks p.i.

Renal casts, however, appeared more frequently in C57BL/6J and Balb/c mice between 8 and 12 weeks p.i.

d. **Ultrastructural localization of amyloid fibrils in host tissues.**

Spleen, liver or kidney sections examined by electron microscopy revealed the presence of randomly oriented non-branching fibrils of variable lengths and 10-12 nm in thickness. These deposits were abundant in the perifollicular areas of the spleen, hepatic parenchyma, walls of blood vessels and hepatic sinusoids and kidney glomeruli, papillae and interstitium. Tissue distribution of amyloid fibrils was comparable to that described earlier by light microscopy. It was also observed that the majority of cells in and around heavy amyloid deposits were mainly cells of the RRS. Amyloid fibrils were identified in the cytoplasmic matrix or in close association with the plasma membrane of these cells (Fig. 1.8). Similar to previous
Table 1.2

Semiquantitative measurements of glomerular and interstitial amyloid deposits in the kidneys at 6, 8 and 12 weeks p.i., from C57BL/6J, Balb/c, CBA, and A/JAX mice infected i.p. with 50 + 10 cysts of E. multilocularis.

<table>
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<tr>
<th>Mouse Strain</th>
<th>Percentage of Glomeruli</th>
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<th>Tubular Papilla</th>
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<tr>
<td></td>
<td>weeks post infection</td>
<td>-ve</td>
<td>+</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>6</td>
<td>66.4</td>
<td>20.2</td>
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<tr>
<td></td>
<td>8</td>
<td>0</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0</td>
<td>10.2</td>
</tr>
<tr>
<td>CBA/J</td>
<td>6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
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<td>41.5</td>
</tr>
<tr>
<td>Balb/C</td>
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<tr>
<td></td>
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<td>13.7</td>
<td>49.1</td>
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<tr>
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<td>8</td>
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</tr>
<tr>
<td></td>
<td>12</td>
<td>90.7</td>
<td>9.2</td>
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</tbody>
</table>

Area covered by amyloid: + (1-25%), ++ (25-75%), +++ (more than 75%)
observations, the majority of the cells were necrotic and damaged. Partial (Fig. 1.8a) or complete dissolution (Fig. 1.8b) of the cell membranes was observed in areas in close association with amyloid fibrils. The nuclear membrane also appeared discontinuous or absent. The DNA material was granular and had lost its dense staining. Furthermore, the intra-cytoplasmic organelles, such as the lysosomal vesicles, were also distorted. Most of the lysosomal membranes were discontinuous or completely fragmented. In a few degenerated cells, membrane-bound vesicles contained structures which resembled AP and amyloid fibrils. Individual subunits of the AP were also seen dispersed among amyloid fibrils, either attached to them or free (Fig. 1.8).

4. DISCUSSION

Results presented here extend previous observations and firmly establish the amyloidogenic potential of alveolar hydatid cyst to induce amyloidosis in various mouse strains, gerbils and humans (Ozeretskovskaya personal communication; Ali-Khan et al. 1982, 1983; Mettler et al., 1982). In C57BL/6J mice amyloid induction was dependent neither on the site nor the dosage of the inoculum (Table 1.1). Both the subcutaneous and intraperitoneal routes of infection were equally effective in inducing amyloidosis and neither the onset nor the progression of amyloid deposition varied when the size of the inoculum was increased from 5 to 50 to 250 cysts.
Electron micrograph of a mononuclear leukocyte in the perifollicular amyloid "ring" in the spleen of a C57BL/6J mouse infected i.p. with 50 ± 10 cysts of E. multilocularis.

(A) Early degenerative changes observed in the cell at 8 weeks p.i. Note the following: (1) the dissolution of certain parts of the plasma membrane in close association with amyloid fibrils (AF) and amyloid P component (AP)-like structures. (2) Intracytoplasmic degradation of lysosomal membranes, (3) intracytoplasmic AF and AP (in a phagosome) and (4) apparent swelling and disruption of the mitochondria. X 36,000.

(B) Final stages of the degenerative events observed in a cell at 12 weeks p.i. Degeneration is much more pronounced: (1) Complete dissolution of the plasma membrane which becomes densely packed with AF (2) autolysis. (3) Intracytoplasmic AF and AP, (4) complete mitochondrial destruction, and (5) complete obliteration of the nuclear membrane. X 36,000.
Of the four mouse strains inoculated with a uniform inoculum of 50 cysts, Balb/c mice consistently showed a larger parasite biomass than C57BL/6J, CBA/J or A/JAX mice. At 6 and 12 weeks p.i. the mean LCM weight in Balb/c mice was 3 to 5-, and 2-fold greater than those isolated at the corresponding time intervals from the other three host strains (Table 1.1). However, despite the relatively uniform susceptibility of C57BL/6J, CBA/J and A/JAX mice to the infection, neither the onset nor the amount of tissue amyloid corresponded with the mean LCM weights (Figs. 1.1 and 1.2). A/JAX hydatid-mice, however, were distinctly and relatively resistant to LCM induced amyloidogenesis. Their spleens did not become positive until 12 weeks p.i. In sharp contrast, 100% of Balb/c spleens and 33% of C57BL/6J spleens became positive for amyloid at 6 weeks p.i. Amyloid fibrils could not be detected in the spleens of CBA/J mice until 8 weeks p.i. Similarly, microscopic examination of Congo red stained sections and quantification of tissue amyloid by morphometric means at different time-intervals confirmed a slow but progressive accumulation of amyloid. Balb/c spleens had the highest and A/JAX spleens the lowest concentration of amyloid (Fig. 1.2). Amyloid resistance in the A/JAX strain is well established and is thought to be related to defective processing of serum amyloid A protein (SAA) at the lysosomal level rather than decreased synthesis of SAA (McAdam and Sipe, 1976; Wohlgethan and Cathcart, 1979). Based on the amount and duration of exposure to casein, A/JAX mice have been shown to require a 3 to 4 fold longer exposure time to amyloid induction than CBA/J,
C57BL/6J or Balb/c strains (Janigen and Druet, 1966; Cohen and Shirahama, 1972). Whether the mechanism of amyloid resistance in A/JAX hydatid-mice is similar to the conventional casein-mouse model remains to be answered. However, data obtained in this study (Chapter 4) will show that the induction period of amyloidosis in A/JAX mice can be shortened dramatically by the transfer of an amyloid enhancing factor-like substance at the time of infection.

The morphological appearance of amyloid deposits in the spleens, livers and kidneys of hydatid infected mice, its pathognomonic greenish birefringence, ultrastructural pattern and dimensions are typical of amyloid fibrils (Fig. 1.8). The association of AP with the fibrillar deposits as seen in EM sections further confirms the nature of the fibrils. Furthermore, degenerative changes that were observed in the cellular components and which accompanied the appearance of the fibrillar deposits were similar to those described for casein-induced amyloidosis (Gueft and Ghidon, 1968). In the next chapter, histochemical and immunohistologic characterization of the fibrillar deposits are described. These studies revealed that the amyloid in hydatid mice is indeed of AA-type.

Except for the focal accumulation of inflammatory cells in the liver, the histopathologic alterations and progression of amyloid deposits in the spleens, kidneys and livers of hydatid-mice were similar to the conventional casein-mouse model. The latter has previously been extensively discussed (Teilum, 1964; Hardt and Ranlov, 1976; Glenner and Page, 1976). The periportal
and centrilobular cuffs of inflammatory cells and those plugging the sinusoids before and after amyloid deposition contrasted both qualitatively and quantitatively between hydatid mouse strains. A/JAX mice, which had minimal amyloid deposits in the liver, showed minimal liver inflammation and the predominant infiltrates were monocytoid cells and lymphocytes. In contrast, relatively heavier granulocytic infiltration in the livers of Balb/c and C57BL/6J mice preceded amyloid deposition and was exacerbated by the progressive accumulation of amyloid (Figs. 1.5b, 1.5d). The latter observations are of significance for two reasons. First, accelerated acquisition of amyloidosis in mice has been correlated with severe neutrophilia and neutrophil destruction (Kisilevsky et al., 1977). More recently, membrane-bound and lysosomal elastase-like serine proteases from monocytoid cells and neutrophils, respectively, have been shown to cleave SAA into small peptides or subunits structurally similar to that of fibrillar AA amyloid (Lavie et al., 1980; Silverman et al., 1982). The significance of these observations is that during certain chronic inflammatory diseases the "breakdown in the normal tissue homeostasis" may favor transformation of the subunits of SAA into amyloid fibrils. Inoculation of alveolar hydatid cysts in mice, either subcutaneously or intraperitoneally, triggers a massive influx of leukocytes at the focus of infection (Ali-Khan and Siboo, 1980; Devouge and Ali-Khan, 1983). Intense and persistent peripheral and tissue neutrophilia and histiocytosis is a hallmark of murine alveolar hydatidosis (Rausch 1954, Ali-Khan 1974; Ali-Khan, 1978).
However, in view of the fact that focal liver inflammation has not been associated with amyloidogenesis in mice infected with casein or infected with *Candida albicans* (Sorensen et. al., 1964; Takahashi, 1977; Savage and Tribe, 1978), it is not possible to say whether heavy amyloid deposition in the livers of Balb/c and C57BL/6J hydatid-mice is an expression of intense focal inflammation or an unrelated event analogous to immune-complex (IC) mediated perisinusoidal and periportal inflammation observed in tropical splenomegaly syndrome (Marsden and Crane, 1976). In this context, it is important to point out that both circulating and tissue deposited IC have been demonstrated in hydatid mice (Ali-Khan and Siboo, 1980, 1983).

Finally, casein-induced amyloidosis in mice, pathologically and pathophysiologically similar to human secondary amyloidosis, has been universally used to study the pathogenesis of amyloidosis. The primary interest of the alveolar hydatid cyst-mouse model in this context is that it provides an appropriate alternative to study the relationship between circulating immune complexes, recurrent inflammation, hypergammaglobulinemia (Ali-Khan and Siboo, 1980, 1982 1983), depressed or delayed hypersensitivity response (Ali-Khan 1978a), involution of thymus and disorganization of lymphoreticular tissues (Ali-Khan, 1978b; Devouge and Ali-Khan, 1983) and secondary amyloidosis. All the above, one time or another, have been incriminated as the pathogenetic factors in the induction of amyloidosis. In addition, it can now be stated with certainty that the present model is predictable and the induction of amyloidosis is virtually 100% in both susceptible and resistant mouse strains.
CHAPTER TWO

CHARACTERIZATION OF AMYLOID PROTEIN FROM MICE INFECTED WITH ALVEOLAR HYDATID CYSTS: ISOLATION, PURIFICATION, CHARACTERIZATION AND AMINO ACID ANALYSIS.
1. INTRODUCTION.

Amyloidosis occurs spontaneously or as a result of recurrent inflammation induced with or without intervention by infectious agents (Cohen 1967; Glenner 1980). Amyloidosis, probably of AA-type, has been reported from patients infected with protozoan or metazoan parasites (Andrade and Rocha 1979; McAdam et al. 1980); the chemical nature of the amyloid deposits were, however, not confirmed. As presented in Chapter 1, amyloid induction in alveolar hydatid cyst (AHC) infected mice of different H-2 background was comparable to that reported in mice against multiple azocasein injections (Janigan and Druet 1966). A/J mice in both instances appeared to be relatively resistant to amyloid induction. In a previous study it was shown that despite cross reactivity between rabbit-anti mouse AA serum and AHC-induced amyloid protein, the latter unlike the casein-induced amyloid was resistant to KMnO₄ -trypsin treatment (Wright et al. 1977; Ali-Khan et al. 1983). This observation raised the question whether the amyloid substance in AHC-mice was chemically similar to that of azocasein-induced amyloid. The literature clearly indicates that histochemical characterization of amyloid deposits in situ can be imprecise and often misleading. In a number of cases both the AA and AL types of amyloid have been isolated from the same patient. Westermark et al. (1976), Cornwell et al. (1977) and Baumal (1979) have shown, using the indirect fluorescent antibody
test, that rabbit anti-mouse protein AA serum cross reacts with amyloidotic spleens obtained from Balb/c mice bearing the MOPC-173 tumor (AL-type) and also from aged SJL/J mice (AS-type). Their findings indicate that amyloid deposits in the same host are not homogeneous, but rather a mixture of two or more types of amyloid proteins (Wright et al. 1977). Two other histochemical methods have been described for the differentiation between AE, AS or AL and AA forms of amyloid depending on their ability to retain green Congo red birefringence following induced proteolysis by trypsin (Romhanyi 1972) or after potassium permanganate treatment (Wright et al. 1974, 1977). Either method shows that the AA-type of amyloid fibrils which are associated with chronic inflammatory disorders are sensitive to these treatments while AL, AE and AS-types are resistant.

A precise biochemical definition of the amyloid and its putative precursor is required for a better understanding of the inducing and pathogenetic processes involved (Harada et al. 1971). Electrophoretic mobility, molecular weight determination, amino acid analysis and amino acid sequencing of the amyloid substance have been used to differentiate between various amyloid proteins (Eriksen et al. 1976; Scheinberg et al. 1976a; Benditt et al. 1979; Eriksen and Benditt 1980; Hoffman and Benditt 1980; Matsumura et al. 1982; Kisilevsky 1983; Hoffman et al. 1984). Several methods for the purification of amyloid fibril-rich concentrates by fractionation have been described: the water extraction method of Pras et al. (1968), the guanidine extraction process (Glenner et al. 1969; Harada et al. 1971)
and the urea-formate method of Eriksen et. al. (1976).

In recent years special interest has been directed toward the development of an experimental animal model which would offer clues to the pathogenesis of amyloidosis (Cohen and Shirahama 1972; Kisilevsky 1983). Much of our present knowledge on secondary amyloidosis emanates from materials isolated from patients or from azocasein-induced AA amyloid and its presumed precursor, serum amyloid A-protein (SAA) from mice (Linke et. al., 1975; Scheinberg et. al., 1976a; Gorevic et. al., 1978; Eriksen and Benditt 1980; Hoffman et. al., 1984). This and other similar observations are based on antigenic similarities and amino acid sequence homologies between these two pathologic proteins (Eriksen et. al., 1976; Gorevic et. al., 1978; Eriksen and Benditt 1980). Recently an antigenically and chemically distinct amyloid protein has been described from senescence-accelerated mice (Scheinberg et. al., 1976a; Matsumura et. al., 1982; Higuchi et. al., 1983). This novel amyloid protein differs from the mouse protein AA in molecular weight and amino acid composition.
2. MATERIALS AND METHODS

a. Animals.

1. Male C57BL/6J mice 6-8 weeks of age were used in this study unless otherwise indicated. They were fed on purina chow and water ad libitum.

2. Rabbits. New Zealand white female rabbits weighing two to three kilograms were purchased from Canadian Breeding Farm, St. Constant, Quebec. Each rabbit was housed individually and fed commercial chow and water ad libitum.

b. Source of amyloid.

1. Alveolar hydatid cyst induced amyloid (AHCA). Sixty mice were infected intraperitoneally with 50 ± 10 cysts of *Echinococcus multilocularis* as has been described previously (see chapter 1). At 12 weeks post-infection (p.i.) mice were sacrificed and their spleens, livers, and kidneys were collected and either processed immediately or frozen at -20°C, until used.

2. Azo-casein induced amyloid (AZCA). Sixty mice were injected subcutaneously daily with 0.3 ml of 10% azocasein in 0.1M NaHCO₃, pH 7.5 (Janigan and Druet 1966). Three weeks later the mice were sacrificed and their spleens, livers and kidneys were collected and
either used immediately or kept frozen at \(-20^\circ C\), until processed. Tissue extracts were monitored at 280 nm to assess the amount of protein dissolved and the extraction process was terminated when the absorbance of the supernatant reached zero.

c. **Extraction of amyloid fibrils**

Amyloid fibrils were extracted from the tissue homogenates either in distilled water, following the method of Pras et al. (1968), or by solubilization in 6M Urea-formic acid solution, pH 3, as has been described by Benditt and Eriksen (1972).

d. **Normal tissue extract.**

Spleens, kidneys and livers from twenty uninfected mice were used for the extraction of tissue proteins described above. The lyophilized tissue extract was used as a control against amyloid preparations.

e. **Gel filtration.**

Lyophilized, water or urea-extracted amyloid were dissolved separately in 5 to 10 ml of 6M Urea in 0.2M NaCl (adjusted to pH 3.0 with formic acid) solution (acid-urea solution). The suspension was kept for 12 hours at 4\(^\circ C\) with continuous stirring. The solubilized amyloid was centrifuged at 16,000 x g for 45 minutes. The supernatant was passed upwards through a 2.5 x 93 cm column of Sephadex G-100 equilibrated with acid urea solution at room temperature or at 4\(^\circ C\). Different peaks identified by absorbance at 280 nm were collected, dialyzed exhaustively
against 0.5% glacial acetic acid using Spectrapor membrane tubing number 3 (molecular weight cut off 3500) and lyophilized. The second fraction which contained most of the amyloid was re-refractionated on a Sephadex G-50 column, (1 x 93 cm; equilibrated with acid-urea), dialized and processed as indicated above. Total protein in each G-100 and G-50 fraction (Figs. 2.1-2.4) was determined by using the BIO-RAD protein assay solution according to the manufacturer’s instructions (BIO-RAD Laboratories, Mississauga, Ontario, Canada).

**f. NaDodSO₄-polyacrylamide gel electrophoresis (SDS-PAGE).**

SDS-PAGE was performed in a cooled Bio-rad protein slab cell 165-1423 following the methods of Laemmli (1970) and Benditt & Erikson (1972). The separating gel of 12% acrylamide and a dimension of 15 cm x 0.75 mm contained the following reagents: 9.8 ml double distilled water (DDW), 7.5 ml of 1.5 M Tris-HCl buffer pH 8.8, 0.6 ml of 10% w/v NaDodSO₄ (SDS) in DDW, 12.0 ml of acrylamide (30% T, 2.67% C), 0.05 ml of 10% ammonium persulfate (APS) prepared fresh and 20 ul of Tetramethylethylenediamine (TEMED); (total volume 30 ml). The separating gel was cast and a thin layer of DDW was layered carefully on top. The gel was allowed to polymerize for 30 to 60 min. or overnight at room temperature.

The stacking gel of 5% acrylamide and a length of 1.5 cm contained the following: 7.6 ml of DDW, 3.75 ml of 0.5 Tris-HCl (pH 6.8), 0.15 ml of 10% SDS, 2.5 ml of acrylamide (30% T, 2.67% C), 0.95 ml 8 M urea, deionized in Rexyn 1-300 (Fisher Scientific
0.05 ml of 10% APS and 10 ul of TEMED; (total volume 15 ml). The stacking gel was allowed to polymerize for 45-60 min.

The sample buffer contained 1 ml of 0.5 M Tris-HCl, pH 6.8, 1 ml of 10% SDS, 1 ml glycerol, 0.1 ml of 0.5% Bromophenol blue as a tracking dye, 1.3 ml of DDW and 0.65 ml of 8 M urea. The electrode (running) buffer (pH 8.3) contained the following: 0.025 M Tris-HCl buffer, 0.192 M glycine and 0.1% SDS.

Lyophilized protein fractions were dissolved (2 mg/ml) in the sample buffer and kept in a boiling water bath for 5 min. Twenty five microliters of the sample containing 25 to 80 ug of protein was applied to each well in the stacking gel and electrophoresed at 5 mA/gel until the sample had entered the gel. Then it was run at 10 mA/gel until the sample had reached the separating gel. For the rest of the run (2-3 hrs), 30 mA/gel was applied. The electrode buffer was used for a maximum of 3 runs or a period of 96 hours.

g. Isoelectric focusing.

For analytical isoelectric focusing, 0.75 mm thick slab gels of a total length of 10 cm were used. The gel contained the following reagents: 0.15 ml of 5% acrylamide (5% T and 3% C), 4 ml of glycerol, 1.9 ml of Pharmalyte (pH range 4 to 6.5 and 3 to 10; Pharmacia Fine Chemicals, Dorval, Que.), 15 ml of DDW, 200 ul of freshly prepared APS (22.8 mg/ml) and 50 ul of TEMED. The gels were cast on silanized glass plates (silane A-174, Pharmacia) in order to covalently link the polyacrylamide to the glass plate. This manipulation facilitates further handling of
the gel during fixation, staining and destaining. For Pharmalyte pH range 4.0-6.5, 0.04M DL-glutamic acid was used as an anolyte and 0.2M L-Histidine as a catholyte. For Pharmalyte pH range 3-10, 0.04M D-L aspartic acid was used as an anolyte and 0.1 M NaOH as a catholyte. The sample buffer contained 0.7 ml of DDW, 0.1 ml of glycerine, 0.1 ml of 0.5% Bromophenol blue and 0.1 ml of 8 M Urea. For isoelectric point estimation of the hydatid-mouse and casein-mouse amyloid proteins both low and high pI standard proteins (Pharmacia Fine Chemicals, Dorval, Que) were used.

Lyophilized protein fractions were dissolved in the buffer (4 mg/ml) at room temperature for 30 min. The samples (10-25 ul) were applied to acetate paper (1.0 x 0.5 cm) which was subsequently applied to the gel 4 cm away from the cathode. Electrophoresis was performed in a LKB flat bed 2117 Multiphore with an LKB-2013 power supply, at 30 watts and a maximum voltage of 2000 volts for 2-3 hrs at current setting of 30 mA. The temperature of the coolant was adjusted so that the temperature in the gel was 15° C ± 2° C at the end of the run. The gels were stained in 0.1% Coommasie brilliant blue G-250 in 25% ethyl alcohol and 5% acetic acid in DDW for 3 hrs and destained in 25% ethyl alcohol and 5% acetic acid in DDW.

h. Preparation of rabbit antisera against hydatid-mouse amyloid (AHCA) and azocasein-mouse amyloid (AZCA).

The lyophilized amyloid (2nd peak, G-100 gel) was degraded in 0.1 M NaOH for 1 hr (1mg/ml) at room temperature as has been described by Pras et. al. (1969). The degraded amyloid fibrils (DAF) were neutralized with 1N HCl, mixed with an equal volume of
complete Freund's adjuvant (CFA) and used as boosters. Initially each rabbit (New Zealand white females weighing 3 kg) received a priming intravenous injection of 0.5 ml of soluble DAF. After 5 days each rabbit received, at multiple sites (intramuscularly and subcutaneously), 2 ml of DAF-CFA. Multiple subsequent boosters were spaced between two to four weeks and continued for at least 6 months. Rabbits were bled from time to time and their sera tested for anti-amyloid antibody against DAF by the Ouchterlony test, or amyloid deposits in tissues, using the indirect immunofluorescence test. The test sera were preadsorbed twice with guinea pig and mouse liver, kidney, and spleen tissue powder (10 mg of tissue powder and 100 ul of serum before use.

**1. Gel diffusion, immunoperoxidase and immunofluorescence techniques.**

**1. Gel diffusion.** The Ouchterlony immunodiffusion test was performed in 1% agarose prepared in barbital buffer, pH 8.6 (Garry et al. 1977). Lyophilized amyloid protein (5 mg/ml) was degraded in 0.1 M NaOH for 2 hr and the pH was adjusted to 8.5 with 1 N HCL. The degraded amyloid was centrifuged at 500 g for 15 min before application to the peripheral wells. Anti-amyloid serum was applied to the central well. The plates were incubated at room temperature for 24 to 48 hr.

Indirect immunofluorescence (IFA) and immunoperoxidase (IP) techniques using rabbit anti-hydatid amyloid (RAHCA) and rabbit anti-azocasein mouse amyloid (RAZCA) sera were used to detect cross-reactivity between the AHCA and AZCA amyloid deposits.
Formalin-fixed, paraffin embedded kidney sections as well as frozen kidney sections from azocasein mice and hydatid-mice were used.

2. Immunoperoxidase (IP) assay.

Deparaffinized alcohol treated sections (4-7μm thick) were placed for 30 minutes in methanol, 0.5% hydrogen peroxide to deplete the endogenous peroxidase. Subsequently, the sections were sequentially incubated for 10 minutes in a 1:20 dilution of normal goat serum for 30 minutes in a 1:50, 1:100 and 1:200 dilution of RAHCA or RAZCA antisera and a 1:250 dilution of goat anti-rabbit IgG serum conjugated with peroxidase. After each incubation period, the slides were washed with Tris-saline buffer for 15 minutes (0.9% NaCl in 10mM Tris-HCl, pH 7.4). Antiserum dilutions were made with this buffer containing 20% goat serum. Following the last incubation and washing, the peroxidase reaction was developed by incubating the slides with the substrate (0.3 mg/ml of 3.3 diaminobenzidine hydrochloride and 0.005% H₂O₂ in 50 mM Tris-buffer, pH 7.5). The reaction was stopped by immersing the slides in Tris-saline buffer.

3. Indirect immunofluorescence technique (IFA).

After deparafinization, tissue sections, 4-7 μm thick, were equilibrated with phosphate-buffered saline (PBS) 0.01 M, pH 7.2. They were then incubated for 30 minutes in 1:25, 1:50, 1:100 or 1:200 dilutions of RAHCA or RAZCA antisera followed by goat IgG serum conjugated with fluorescein for an additional 30
minutes at 4°C. After each incubation, the slides were washed with ice cold PBS for 15 minutes (3 changes). The sections were mounted in buffered glycerol (9 parts glycerol and 1 part carbonate buffer 0.5 M, pH 9.3) and examined with a Wild Leitz dialux 20 fluorescence microscope equipped with FITC filter.

The controls for the IP and IFA tests were designed to detect non specific binding of IgG to the amyloid protein and normal tissue components. It consisted of incubating spleen, liver and kidney tissue sections of hydatid mice and age-matched normal mice with normal rabbit serum followed by goat anti-rabbit IgG serum conjugated to fluorescein or peroxidase. Specificity of RAZCA against hydatid induced amyloid deposits and RAHCA against azocasein induced amyloid deposits was determined by adsorbing the antisera against heterologous amyloid. One milligram of the purified and lyophilized amyloid proteins were incubated with 0.5 ml of the heterologous antisera for 30 min at 37°C and overnight at 4°C. Test sera were adsorbed at least twice with amyloid protein before use in IFA and IP techniques.

**J. Electrophoretic transfer of protein to nitrocellulose paper (NCP).**

The method of Towbin et al. (1979), was followed with slight modification for the characterization of polypeptides transferred from polyacrylamide gels to NCP. Samples containing crude and G-100 fractions were first subjected to SDS-PAGE (as described earlier) and then transferred to NCP. The electrophoretic blots were then incubated in 3% bovine serum albumin (BSA) in Tris-saline buffer (0.9% NaCl in 10 mM Tris-HCl,
pH 7.4) for 1 hr at 40°C in a circulating water bath. The NCP blots were rinsed in Tris-saline buffer and incubated with the appropriate antisera (Rabbit anti mouse hydatid amyloid, RAHCA, or Rabbit anti-mouse casein amyloid, RAZCA, preadsorbed with normal mouse and guinea pig tissue powder). The final dilution of the antiserum used was 1:50 in Tris-saline buffer. After incubating for 1 hr at room temperature or at 4°C overnight, the NCPs were washed with Tris-saline buffer (5 changes for 30 min.) and reincubated for an additional 1 hr at room temperature with goat anti-rabbit IgG serum conjugated with peroxidase (1:250 dilution in Tris-saline buffer; United States Biochemicals, USA). Following the incubation, the NCP sheets were washed with saline-Tris buffer (5 changes for 30 min.) and the peroxidase activity was detected by incubating the transfer blots with the substrate (0.3 mg/ml of 3.3 diaminobenzidine hydrochloride and 0.005% fresh H2O2 in 50 mM Tris buffer, pH 7.5). The intensity of the reaction was stopped by immersing the sheets in the buffer and then in DDW.

k. Potassium permanganate treatment and trypsin digestion.

Seven millimicron thick, paraffin embedded and formalin-fixed liver, spleen, and kidney sections were used. The tissues were obtained from C57BL/6J, A/JAX, CBA and Balb/c mice infected 12 weeks before with E. multilocularis cysts (50 ± 10 cysts and C57BL/6J mice treated with azocasein (10% azocasein, 0.5 ml/day/mouse for 3 weeks). The sections were processed in xylene, descending grades of alcohol and finally brought to
water. The slides were immersed in various concentrations of potassium permanganate (KMnO₄) solution (0.5, 2.5 or 5% in 0.3% H₂SO₄) for 2.5 minutes and then in 0.5 or 5% oxalic acid in DDW until completely decolorized as has been described by Wright et al. (1977). The sections were rinsed in DDW and incubated with trypsin-phosphate buffer (1, 5, 10, or 100 mg trypsin/100 ml of 0.15 M phosphate buffer, pH 7.4; 10,000-13,000 BAEE units/mg of trypsin) for 1.5 hr at 37°C in a circulating water bath. For control purposes several sections were incubated in the buffer alone for the same period of time. The sections were then stained with Congo red as has been described earlier (Puchler et al., 1962).

1. Molecular weight estimation.

Molecular weight determinations of the purified amyloid were carried out by SDS-PAGE using molecular weight markers ranging from 14 Kd to 205 Kd (Sigma Biochemicals, St. Louis, MO, U.S.A.). Rf values were used for the molecular weight estimation of the amyloid protein.

m. Protein determination.

The BIO-RAD protein assay was used in order to estimate the amount of protein in solutions and tissue extracts. The assay was performed according to the manufacturer's suggestions. Essentially, the dye reagent was diluted with DDW (1:4), filtered through Whatman number 1 filter paper and mixed with the protein sample solution (100 ul/5 ml of the diluted dye reagent). The mixture was allowed to stand at room temperature for 10 minutes
and then the absorbance was measured at 595 nm in a spectrophotometer (spectronic 200 UV, Shimadzu, Bausch and Lomb). A protein standard curve was prepared using bovine plasma gamma globulin or bovine plasma albumin (BSA).

n. Amino acid analysis.

Types DC-4A (lot no. 750) and DC-5A (lot no. 746) cation exchange spherical resins, sized 8.0 x 0.5 mm and 6.0 x 0.5 mm respectively, were purchased from Dionex Chemical Co., Sunnyvale, California. Dowex 50W-X8 (200-400 mesh) and AG5 01-X8D resins were purchased from Bio-Rad Laboratories, Richmond, California. Dl-ornithine (5-aminonorvaline) was purchased from Schwarz/Mann, Orangeburg, New York. The internal standard amino acids were obtained as follows: norleucine and L-2-amino-3 guanidino-propionic acid from Pierce Chemical Co., Rockford, Illinois and 3-nitro-L-tyrosine from Aldrich Chemical Co., Milwaukee, Wisconsin. The highly pure citrate buffers (pH 3.28, 0.20 M; pH 4.25, 0.20 M; pH 6.40, 1.0 M), sample dilution buffer (pH 2.2, 0.2 M) and ninhydrin reagent kit recommended for high-sensitivity single microcolumn standard analysis were purchased either as concentrates or ready-to-use solutions from Beckman Instruments Inc., Palo Alto, California. The chemicals and reagents employed for methylated basic amino acid determinations were purchased from Pierce Chemical Co., Rockford, Illinois. High purity water used in buffers and reagents were prepared by the procedures described by Ganzi (1984). The unusual amino acid calibration standards employed for peak identification and standardization of
the instrument were prepared as described by Zarkadas (1979).

Amino acid analyses were carried out with an updated Beckman amino acid analyzer (equivalent to Beckman Spinco model 121MB). Duplicate amyloid samples (0.5 mg) were hydrolyzed at 100°C for 12, 18, 24, 48, 72, and 96 hr as described by Moore and Stein (1963) and Hare (1977). Standard amino acid analyses of individual samples (1.0-5.0 ug) were performed in duplicate on a 0.28 x 22.5 cm micro-bore column of Dionex type DC-5A resin using the recommended Beckman microcolumn elution buffers A, B and C, by a high sensitivity method (unpublished data, Zarkadas). Similarly, methionine and cysteine were determined in separate amyloid samples (0.5 mg) as their oxidation products by the performic acid procedure of Moore (1963).

Tryptophan in amyloid samples (0.1-0.5) was determined separately after alkaline hydrolysis by an improved chromatographic procedure (Zarkadas et al. 1982). Samples were eluted from a 0.28 x 17 cm microbore column of Dionex type DC-5A resin with 0.21 M sodium citrate buffer (pH 5.125) containing 2-propanol (1 % v/v) at 51°C and a flow rate of 7.2 ml/hr and 600 p.s.i. Elution times for 3-nitrotyrosine and tryptophan were 19.95 and 39.50, respectively.

The determination of all methylated basic amino acids and related compounds in amyloid was carried out with concentrated acid hydrolysates (equivalent to 100-200 ug protein) by the accelerated single-microcolumn methods described previously (Zarkadas, 1979) so that peaks adequate for these components could be obtained.
3. RESULTS

a. Extraction of tissue amyloid.

Amyloid fibrils from hydatid-mouse tissues were extracted either by solubilization in distilled water or denaturation in 6 M acid-urea solution. Tissue extracts were monitored at 280 nm to assess the amount of protein dissolved. The extraction process was ended when the absorbance of the last washing approached zero. Either method extracted considerable amounts of amyloid from the tissue. Amyloid deposits were still present in the sediment of the last washing in water as assessed by Congo red binding and green birefringence when viewed under polarized light. When the tissue pellet after the water extraction was centrifuged and examined microscopically it showed four layers of different coloration as has been described earlier (Glenner et al., 1971). Amyloid fibrils were distributed equally in the third and fourth layers. The first and second layers contained considerably less amyloid. When this sediment was subjected to urea extraction, amyloid was detected in the supernatant. At this stage it was not possible to assess the residual amyloid left in the tissue sediment, since after urea treatment amyloid loses its congophilic birefringence under polarization microscopy and its fibrillar appearance by electron microscopical examination. Water soluble amyloid preparations exhibited all the physical characteristics of amyloid. For instance it bound Congo
red, sedimented at 100,000 x g in 2 hr, aggregated in the presence of a trace amount of NaCl, and appeared opalescent when the amyloid suspension (1-2 mg/ml of protein) was left at 4°C for 7-10 days (Pras et al. 1968).

As indicated in the materials and methods section the extraction of tissue amyloid with acid-urea solution proved to be less cumbersome; the second incubation mixture (acid-urea) extracted an additional quantity of amyloid from the lyophilized tissue.

b. Purification by gel filtration.

Chromatography of acid-urea, (Figs. 2.1 & 2.3) or water soluble amyloid (Fig. 2.2) on Sephadex G-100 revealed 4 major peaks. Peaks 1, 2 and 4 were similar in both cases; peak 3 was more prominent in the acid-urea extract. Treatment of the tissue sediment with acid urea for 12 hrs at 4°C subsequent to water extraction yielded amyloid which was apparently not soluble in water. Its chromatographic pattern was similar to that in Fig. 2.1; peak 2 was reduced and peaks 3 and 4 were enlarged. When these fractions were dialyzed against 0.5% acetic acid the fourth fraction was lost. It did not show any absorbance at 280 nm. Apparently peak 4 contained polypeptides of molecular weights less than 3500.

Chromatograms of acid-urea extracts of spleens, livers and kidneys obtained from azocasein treated mice were similar to those of water or acid-urea soluble hydatid-amyloid (Fig. 2.3). Four peaks were detected. However, peaks 2 and 3 were less
Figure 2.1

Sephadex G-100: ascending chromatography in urea-formate buffer of urea-extracted alveolar hydatid cyst-induced amyloid. Sample size 50 mg of freeze-dried crude amyloid in 5 ml of elution buffer; flow rate 20 ml/hr; fraction volume 5 ml/tube. Eluted fractions were pooled as indicated in the figure. The distribution of protein in each fraction is shown at the top of the figure.

Figure 2.2

Sephadex G-100: ascending chromatography in urea-formate buffer of water-extracted alveolar hydatid cyst-induced amyloid. Sample size 50 mg of freeze-dried crude amyloid in 5 ml of elution buffer; flow rate 20 ml/hr; fraction volume 5 ml/tube. Eluted fractions were pooled as indicated in the figure. The distribution of protein in each fraction is shown at the top of the figure.
Figure 2.3

Sephadex G-100: ascending chromatography in urea-formate buffer of acid urea-extracted AA-amyloid. Sample size 50 mg of freeze-dried crude amyloid in 5 ml of the elution buffer; flow rate 20 ml/hr; fraction volume 5 ml/tube; eluted fractions were pooled as indicated in the figure. The distribution of protein in each fraction is shown at the top of the figure.

Figure 2.4

Sephadex G-50: ascending chromatography of peak 2 from the G-100 gel. Sample size 25 mg of freeze-dried amyloid in 3 ml of the elution buffer; flow rate 20 ml/hr; fraction size 2.5 ml/tube. Eluted fractions were pooled as indicated in the figure. The distribution of protein in each fraction is shown at the top of the figure.
separated and were joined by their trailing shoulders.

The distribution of protein in each fraction obtained from ARCA and AZCA is shown in Figs. 2.1, 2 & 3. Between 18 to 30% of the total protein eluted with the amyloid peak (peak 2). However, the water extraction method (Fig. 2.2) yielded relatively more contaminant-free amyloid protein from the homogenized tissue than the acid-urea extraction method as judged by SDS-PAGE. Since G-100 peak 2 contained most of the amyloid protein, it was rechromatographed on Sephadex G-50 gel. It eluted as a single peak which was subdivided into 3 fractions (Fig. 2.4). The SDS-PAGE analysis revealed that fraction 1 contained most of the contaminants, fraction 2 had most of the amyloid protein, represented as the doublet, and fraction 3 contained trace amounts of the amyloid. Therefore, as indicated in the materials and methods, the 2nd fraction was used for immunization and further characterization of the AHCA protein.

c. SDS-PAGE and immunoblotting.

Figure 2.5 illustrates the SDS-PAGE patterns of the normal tissue extract and the crude AHCA and AZCA, and their Sephadex G-100 and G-50 gel fractions. Along with other proteins, a fast moving doublet appeared in both the crude (lanes 1,6) and the purified (lanes 2-4) AHCA and AZCA preparations. The doublet was absent from the normal tissue extract (lane 5). It was identified as the amyloid protein by blotting the SDS-PAGE gel on NCP (see below) and treating the blot with RAZCA and goat anti-rabbit IgG conjugated to peroxidase (Fig. 2.6). The doublet in
Figure 2.5

SDS-PAGE of AHCA and AZCA: (1) crude AHCA, (2) 10 ug of purified AHCA, (3) 10 ug of purified AZCA, (4) 25 ug of peak 2 from G-100 purified AHCA, (5) normal tissue extract, (6) crude AZCA.

Figure 2.6

Immuno-electrophoretic blotting of crude and purified hydatid amyloid from SDS-PAGE, using rabbit anti serum against azocasein induced amyloid protein. (A) molecular weight markers (B) crude AHCA, (C) 50 ug normal tissue extract, (D) 50 ug purified AHCA, (E) 25 ug purified AHCA, (F) 25 ug normal tissue extract, (G) crude AZCA, and (H) 25 ug purified AZCA.
the crude AHCA and its fractions bound two other anti-mouse AA amyloid sera (gifts from Dr. Nils Eriksen, Dept. of Pathology, University of Washington, Seattle and Dr. Robert Kisilevsky, Dept. of Pathology Queen's University, Ontario) and showed a positive reaction by IP. Similar to the AHCA most of the AZCA (AA-amyloid) was present in peaks 2 and 3, of the G-100 fractions as determined by SDS-PAGE analysis (Fig 2.5). The doublet in AZCA comigrated along with the doublet in ACHA and in immunoblotting experiments it specifically bound to the RAZCA (Fig. 2.6). The RAZCA did not bind to the molecular weight standards (lane A) or normal tissue extracts (lanes C, F), but showed specific binding to the doublets present either in the crude AHCA and AZCA (lanes B, G) or their G-50 fractions (lanes D, E, H). The resolution of the NCP-transferred doublets, however, could not be intensified. Similar resolution was obtained when increasing amounts of amyloid samples were electrophoresed and blotted on NCP or even incubated for longer periods in the electric field. The capacity of the NCP seems to be limited to a maximum of 10-20 ug of protein per band.

d. Molecular weight and pI estimation.

On SDS-PAGE the approximate molecular weight of the AHCA was found to be between 8.77 and 9.9 kd. On thin layer polyacrylamide gel isoelectric focusing the purified AHCA (Fraction 2, Fig. 2.4) resolved into two adjacent bands within the pH range of 5.1 and 5.3 (Fig. 2.7). These bands occupied a position which was comparable to the band formed by B-
Figure 2.7

Analytical isoelectric focusing of purified AHCA (track 3) and AZCA (tracks 2, 4) on thin-layer polyacrylamide gel. The pI standards (track 1) are from top to bottom: Amyloglucosidase (pI 3.5), Soybean trypsin inhibitor (pI 4.55), B-lactoglobulin A (pI 5.2), Bovine carbonic anhydrase B (pI 5.85), Human carbonic anhydrase B (pI 6.55), Horse myoglobin acid band (pI 6.85), Horse myoglobin basic band (pI 7.35), Lentil lectin acidic band (pI 8.15), Lentil lectin intermediate band (pI 8.45), and Lentil lectin basic band (faint) (pI 8.65).
lactoglobulin A (pI 5.2). The protein band with a pI of 5.3 consistently appeared faint. The purified AZCA resolved into one single band and it comigrated with the bovine carbonic anhydrase B (pI 5.85).

e. *Sensitivity of hydatid-mouse amyloid to KMnO₄ and trypsin.*

Treatment of AHCA deposits in tissue sections from C57BL, A/J, CBA and Balb/c strains with KMnO₄ and trypsin followed by Congo red staining showed that AHCA deposits were resistant to digestion. No significant decrease in the intensity of green birefringence was observed even with varying concentrations of KMnO₄ (0.5, 2.5 and 5%). oxalic acid (0.5 and 5%) and trypsin (1, 4, 10 and 100 mg) were used in the test solution (for details see materials and methods). On the other hand, AZCA deposits were completely susceptible to digestion. No deposits were detected after treatment with KMnO₄ and trypsin. It is interesting to note that KMnO₄ treatment of AA-protein (AZCA) without trypsin resulted in the loss of its Congo red affinity and green birefringence. Such treatment had no effect on AHCA.

f. *Antigenic cross-reactivity between AHCA and AZCA.*

Antisera raised against AHCA or AZCA showed considerable cross reactivity in IP and IFA tests against heterologous amyloid. Both antisera showed intense fluorescence or peroxidase staining of amyloid deposits in the renal glomeruli and hepatic sinusoids (Fig. 2.8). RAZCA serum preadsorbed with lyophilized AHCA or AZCA failed to react with either AZCA or AHCA deposits in tissue sections. In contrast, the RAHCA serum preadsorbed with
Figure 2.8
Liver section, C57BL/6J mouse, 12 weeks p.i., immunoperoxidase stain. The amyloid protein was stained with RAZCA and goat anti-rabbit IgG serum conjugated to peroxidase. Note the intense staining of the amyloid deposits.

Figure 2.9
Ouchterlony gel diffusion plate showing the pattern of precipitation bands between (1) serum obtained from mice 12 hours after azocasein injection, (2) serum obtained from AHC-infected mice 8 weeks p.i. (3) DAF (AHCA), (4) DAF (AZCA), (5) normal mouse serum, (6) normal tissue extract, and (central well) rabbit serum against AA-type of amyloid (RAZCA) adsorbed with normal mouse and guinea pig tissue.
lyophilized AZCA retained residual IFA reactivity against AHCA deposits in the renal glomeruli, papillae and interstitium. Figure 2.9 shows the results of a typical Ouchterlony immunodiffusion test. A single precipitation band appeared between DAF (AHCA and AZCA) and RAZCA preadsorbed with guinea pig and normal mouse tissues. These bands showed a reaction of identity with the bands formed between RAZCA and sera from azocasein-stimulated or AHC-infected mice. No lines were detected between the normal tissue extract or normal mouse serum (Fig. 2.9).

g. Amino acid composition and molecular weight estimation.

Table 2.1 presents the mean values and standard errors of the amino acid composition of the purified AHC-induced amyloid protein (Fig. 2.4 fraction 2). The data represents the average values of triplicate determinations obtained from duplicate 12, 18, 24, 72 and 96 hr hydrolysates. The means and standard errors for serine, threonine and tyrosine represent the regression values extrapolated to zero time of hydrolysis (Sanger and Hrempson, 1963). The values for valine, isoleucine, leucine and phenylalanine are averages of data from 48, 72, and 96 hr hydrolysates.

The recovery of cysteine as cysteic acid and methionine as a sulfone were calculated in proportion to the yields obtained by the performic acid treatment of standard solutions of the amino acids and relative to the alanine and leucine present in the sample. The mean values are based on the actual protein content
Table 2.1

The amino acid composition (Mean $C_1$, S.E.M.), frequencies ($a_f$), residue number ($n_f$) and nearest integer ratios ($l_f$) of amyloid protein from alveolar hydatid cyst infected C57BL/6J mice.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Number of Determinations</th>
<th>Mean¹ (mol/10 µg protein) $C_f \pm$ S.E.M.</th>
<th>Mole¹ fraction $a_f$</th>
<th>Residue number $n_f \pm$ S.E.$n_f$</th>
<th>$k_{best} \times n_f$² (r = 0.859; r = 0.48515)</th>
<th>Nearest² integer $l_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>33</td>
<td>7.590 ± 0.068</td>
<td>0.0809</td>
<td>7.020 ± 0.063</td>
<td>5.99 ± 0.48515</td>
<td>6</td>
</tr>
<tr>
<td>Thr</td>
<td>33</td>
<td>5.043 ± 0.048</td>
<td>0.0538</td>
<td>4.664 ± 0.044</td>
<td>3.98 ± 0.48515</td>
<td>4</td>
</tr>
<tr>
<td>Ser</td>
<td>33</td>
<td>6.169 ± 0.075</td>
<td>0.0658</td>
<td>5.706 ± 0.069</td>
<td>4.87 ± 0.48515</td>
<td>5</td>
</tr>
<tr>
<td>Glu</td>
<td>33</td>
<td>9.999 ± 0.095</td>
<td>0.1067</td>
<td>9.248 ± 0.088</td>
<td>7.89 ± 0.48515</td>
<td>8</td>
</tr>
<tr>
<td>Pro</td>
<td>33</td>
<td>4.951 ± 0.192</td>
<td>0.0528</td>
<td>4.579 ± 0.177</td>
<td>3.91 ± 0.48515</td>
<td>4</td>
</tr>
<tr>
<td>Gly</td>
<td>33</td>
<td>8.670 ± 0.119</td>
<td>0.0925</td>
<td>8.019 ± 0.110</td>
<td>6.64 ± 0.48515</td>
<td>7</td>
</tr>
<tr>
<td>Phe</td>
<td>33</td>
<td>8.411 ± 0.132</td>
<td>0.0897</td>
<td>7.799 ± 0.112</td>
<td>6.64 ± 0.48515</td>
<td>7</td>
</tr>
<tr>
<td>Val</td>
<td>14</td>
<td>6.492 ± 0.077</td>
<td>0.0692</td>
<td>6.004 ± 0.071</td>
<td>5.12 ± 0.48515</td>
<td>5</td>
</tr>
<tr>
<td>Met</td>
<td>33</td>
<td>1.477 ± 0.053</td>
<td>0.0157</td>
<td>1.356 ± 0.049</td>
<td>1.16 ± 0.48515</td>
<td>1</td>
</tr>
<tr>
<td>Ile</td>
<td>14</td>
<td>4.568 ± 0.117</td>
<td>0.0487</td>
<td>4.225 ± 0.108</td>
<td>3.60 ± 0.48515</td>
<td>4</td>
</tr>
<tr>
<td>Leu</td>
<td>14</td>
<td>7.610 ± 0.153</td>
<td>0.0812</td>
<td>7.038 ± 0.141</td>
<td>6.00 ± 0.48515</td>
<td>6</td>
</tr>
<tr>
<td>Tyr</td>
<td>33</td>
<td>2.822 ± 0.061</td>
<td>0.0301</td>
<td>2.610 ± 0.056</td>
<td>2.23 ± 0.48515</td>
<td>2</td>
</tr>
<tr>
<td>Phe</td>
<td>14</td>
<td>2.927 ± 0.065</td>
<td>0.0312</td>
<td>2.707 ± 0.060</td>
<td>2.31 ± 0.48515</td>
<td>2</td>
</tr>
<tr>
<td>Lys</td>
<td>33</td>
<td>7.792 ± 0.356</td>
<td>0.0831</td>
<td>7.207 ± 0.329</td>
<td>6.15 ± 0.48515</td>
<td>6</td>
</tr>
<tr>
<td>His</td>
<td>33</td>
<td>2.855 ± 0.194</td>
<td>0.0304</td>
<td>2.641 ± 0.179</td>
<td>2.25 ± 0.48515</td>
<td>2</td>
</tr>
<tr>
<td>Arg</td>
<td>33</td>
<td>5.433 ± 0.206</td>
<td>0.0579</td>
<td>5.025 ± 0.191</td>
<td>4.29 ± 0.48515</td>
<td>4</td>
</tr>
<tr>
<td>Cys</td>
<td>4</td>
<td>0.902 ± 0.022</td>
<td>0.0096</td>
<td>1.077 ± 0.023</td>
<td>0.92 ± 0.48515</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>93.711 ± 1.000</td>
<td>86.915</td>
<td>74.16 ± 76</td>
<td></td>
<td>74</td>
</tr>
</tbody>
</table>

¹The protein content, mole fraction ($a_f$), weight equivalent (WE) and conversion factor ($F$) in each hydrolysate, were calculated according to Horstmann's (1979) method.

²Determinations of the residue numbers ($n_f \pm S.E.n_f$), the goodness of fit, $r$, to integral amino acid residue numbers ($l_f$) as a function of the scaling factor, $k_{best}$, equivalent to a mol. wt. of 8,018.14 daltons for amyloid, were carried out by the computer-assisted method of Hay et al. (1974).
in individual hydrosylate samples and were calculated by Horstman's procedures. The data shows high reproducibility with a SEM of less than 0.25% for all amino acids, except for lysine which showed a slightly higher error. The large number of determinations, as listed in Table 2.1 were necessary to ensure low coefficients of variation (2%) for any given amino acid residue so that the computer-assisted method of Hoy et al. (1974) and Black and Hogness (1969) could be applied for determining both the nearest integer ratios (i.e. frequencies) of amino acid residues in AHCA and a minimum molecular weight for this protein (Table 2.2). These methods yielded a total of 74 residues per amyloid molecule and a molecular weight of 8,018 which is slightly lower than the value (8,700-9,900) found by the SDS-PAGE method. In addition, glucosamine (0.427 mole/mole of amyloid), galactosamine (0.119 mole/mole of amyloid) and trace amounts of methylated amino acids and a number of ninhydrin positive materials were also detected in the analysis of AHCA (Table 2.2).

Table 2.3 compares the amino acid composition of AHC-induced amyloid protein with those induced by azocasein, C. albicans, or developed spontaneously in senile mice. Despite differences in the amino acid composition between different amyloid proteins, aspartic, glutamic, glycine and alanine were present in high proportions in all the amyloid proteins. The proportions of valine, leucine and lysine were relatively low in azocasein, and C. albicans-induced murine amyloid proteins than in those found in other murine amyloids including the present sample. Tryptophan was detected only in trace amounts in the AHCA.
Table 2.2

The methylated basic amino acids, amino sugars and unidentified ninhydrin positive components of amyloid protein from alveolar hydatid cyst infected C57BL/6J mice.

<table>
<thead>
<tr>
<th>Amino acid (AA)</th>
<th>Mean¹ (nmol AA/10 µg protein ± S.E.M.)</th>
<th>Mole¹ fraction</th>
<th>Mole AA/AM fraction (mol%)</th>
<th>wt x 8.018 kdal²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine, GlCN</td>
<td>0.533 ± 0.115</td>
<td>0.005622</td>
<td>0.427</td>
<td></td>
</tr>
<tr>
<td>Galactosamine, GaHN</td>
<td>0.149 ± 0.037</td>
<td>0.001572</td>
<td>0.119</td>
<td></td>
</tr>
<tr>
<td>N⁵-methyl-L-lysine, Lys(He)</td>
<td>0.0252 ± 0.0039</td>
<td>0.000268</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>N⁶-dimethyl-L-lysine, Lys (He₂)</td>
<td>0.0001 ± 0.00010</td>
<td>0.000051</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>N⁶-trimethyl-L-lysine, Lys (He₃⁺)</td>
<td>0.0233 ± 0.0081</td>
<td>0.000091</td>
<td>0.0167</td>
<td></td>
</tr>
<tr>
<td>W-N⁵,N⁶-dimethyl-L-arginine, Arg(He)</td>
<td>0.01535 ± 0.0002</td>
<td>0.000180</td>
<td>0.0123</td>
<td></td>
</tr>
<tr>
<td>W-N⁵,N⁶-dimethyl-L-arginine, Arg(He₂)</td>
<td>0.00192 ± 0.00010</td>
<td>0.000020</td>
<td>0.0015</td>
<td></td>
</tr>
<tr>
<td>W-N⁶-methyl-L-arginine, Arg(He)</td>
<td>0.00135 ± 0.0005</td>
<td>0.000014</td>
<td>0.0011</td>
<td></td>
</tr>
<tr>
<td>Unknown (73.69)</td>
<td>0.0409 ± 0.0001</td>
<td>0.000435</td>
<td>0.0328</td>
<td></td>
</tr>
<tr>
<td>Unknown (131.29)</td>
<td>0.0464 ± 0.0005</td>
<td>0.000493</td>
<td>0.0372</td>
<td></td>
</tr>
<tr>
<td>Unknown (140.40)</td>
<td>0.15527 ± 0.00029</td>
<td>0.00165</td>
<td>0.1245</td>
<td></td>
</tr>
<tr>
<td>Unknown (148.58)</td>
<td>0.0071 ± 0.0009</td>
<td>0.00075</td>
<td>0.0057</td>
<td></td>
</tr>
<tr>
<td>Unknown (203.05)</td>
<td>0.0004 ± 0.0002</td>
<td>0.000181</td>
<td>0.01364</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.0793</td>
<td>0.012096</td>
<td>0.8774</td>
<td></td>
</tr>
</tbody>
</table>

¹The protein content and mole fraction (x₁) in individual hydrolysates were calculated according to Horstmann's (1979) method.

²A mol. wt of 8.0181 kdal for amyloid was determined by the method described by Hoy et al. (1974).

³Concentration of unknowns was estimated using the 5-hydroxyllysine factor, and the number in brackets indicate elution time in min. The unknowns, methylated lysines and N-methyl-arginines were determined by the method of Zarkadas (1979; 1978).
Table 2.3

A comparison of the amino acid composition of amyloid proteins from different strains of mice known to be susceptible to amyloidosis. Expressed as number of residues per 1000 residue.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Present studies</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp</td>
<td>83.4 ± 0.75</td>
<td>85</td>
<td>96</td>
<td>116</td>
<td>108</td>
<td>104</td>
</tr>
<tr>
<td>Thr</td>
<td>55.4 ± 0.53</td>
<td>52</td>
<td>74</td>
<td>26</td>
<td>27</td>
<td>31</td>
</tr>
<tr>
<td>Ser</td>
<td>67.6 ± 0.32</td>
<td>65</td>
<td>84</td>
<td>37</td>
<td>52</td>
<td>67</td>
</tr>
<tr>
<td>Glu</td>
<td>109.9 ± 1.04</td>
<td>116</td>
<td>188</td>
<td>133</td>
<td>126</td>
<td>127</td>
</tr>
<tr>
<td>Pro</td>
<td>54.4 ± 2.11</td>
<td>53</td>
<td>42</td>
<td>21</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Gly</td>
<td>95.3 ± 1.31</td>
<td>85</td>
<td>63</td>
<td>149</td>
<td>148</td>
<td>143</td>
</tr>
<tr>
<td>Ala</td>
<td>92.4 ± 4.75</td>
<td>83</td>
<td>105</td>
<td>139</td>
<td>132</td>
<td>127</td>
</tr>
<tr>
<td>Val</td>
<td>71.3 ± 0.85</td>
<td>57</td>
<td>45</td>
<td>19</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>Met</td>
<td>16.2 ± 0.58</td>
<td>20</td>
<td>15</td>
<td>29</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>Ile</td>
<td>50.2 ± 1.28</td>
<td>40</td>
<td>25</td>
<td>26</td>
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<tr>
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<td>87</td>
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<tr>
<td>Tyr</td>
<td>31.0 ± 0.67</td>
<td>31</td>
<td>14</td>
<td>40</td>
<td>41</td>
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<tr>
<td>Phe</td>
<td>32.2 ± 0.71</td>
<td>46</td>
<td>64</td>
<td>91</td>
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<tr>
<td>Lys</td>
<td>85.6 ± 3.58</td>
<td>81</td>
<td>57</td>
<td>53</td>
<td>60</td>
<td>63</td>
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<tr>
<td>His</td>
<td>31.4 ± 2.13</td>
<td>23</td>
<td>20</td>
<td>29</td>
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<tr>
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<td>59.7 ± 2.25</td>
<td>54</td>
<td>30</td>
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<tr>
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<tr>
<td>Gln</td>
<td>5.9 ± 1.26</td>
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</table>

Total 1029.7 1001 999 1002 1000 1001

(1) AHCA
(A) Age associated amyloid. Scheinberg et al. (1976a)
(B) Amyloid from senile mice. Matsumura et al. (1982)
(C) Casein-induced amyloid. Scheinberg et al. (1976a)
(D) Candida albicans-induced amyloid. Eriksen et al. (1976)
(E) Azocasein-induced amyloid. Eriksen et al. (1976)
4. Discussion

Several methods have been used in the past for the recovery of amyloid proteins from tissues (Pras et al. 1968; Glenner et al. 1971; Benditt and Ericksen, 1973). All the procedures yield considerable amounts of reasonably pure preparations of amyloid fibrils. The acid-urea method has been claimed to give a more purified preparation but with a somewhat lower yield than the water extraction method (Benditt and Ericksen, 1972). In order to compare the data in this study with the published reports on mouse amyloid protein both the extraction method of Pras et al. (1968) and Benditt and Ericksen (1972) for the isolation of hydatid-mouse amyloid have been used. The water extraction method yielded approximately twice the amount of AHCA as compared to the acid-urea extraction method (Figs. 2:1, 2 & 3). Electron microscopical examination of water extracted amyloid showed the typical fibrillar appearance and arrangement of purified amyloid preparations. We are uncertain whether the AHCA has greater solubility in water than in acid-urea solution or the tissues which were treated with water had relatively more amyloid deposits than the acid-urea-treated tissues. Crude amyloid extract from each preparation showed in excess of 15 protein bands on SDS-PAGE. Following the treatment of purified amyloid preparations with urea and the examination by electron microscopy revealed the absence of the typical fibrillar structure.

The physico-chemical characteristics of amyloid proteins
that are associated with chronic inflammation in humans are well
documented but no data is available on the biochemical features
of amyloid deposits which develop as a result of chronic
parasitic infections. Essentially, three chemically and
antigenically different types of murine amyloid proteins have
been identified (Table 2.3). Protein AA was induced either by
azocasein or C. albicans and the degraded amyloid was shown to be
antigenically similar to the azocasein-induced SAA (Benditt and
Eriksen, 1972). Recently Hoffman et. al. (1984) have introduced
the possibility of a precursor-product relationship between apo-
SAA$_2$ and murine protein AA. These two pathological proteins were
shown to have identical NH$_2$-terminal amino acid sequences. The
other two amyloid proteins from senile mice differed from each
other in their amino acid composition and failed to react against
anti-mouse AA serum (Matsumura et. al. 1982). In the present
study, data are presented from azocasein, and AHCA-induced amyloid
preparations from C57BL/6J mice. Before discussing the results
of this chapter three reservations must be made. First, the
amyloid protein studied here constitute only the soluble part of
the amyloid fibrils either in water or acid-urea solution.
Second, no distinction has been made between amyloid deposits
from different tissues (kidneys, livers and spleens were pooled
for the extraction of amyloid). Third, although AHCA protein
develops in several strains of mice infected with alveolar
hydatid cyst (see chapter-1) amyloid fibrils in the current study
were isolated and purified from C57BL/6J mice.

The chromatographic profiles of AHCA and AZCA in G-100 and
G-50 gel columns and three SDS-PAGE patterns were indistinguishable. Both these amyloid proteins preceded the tracking dye and appeared as a doublet (Fig. 2.5), which was identical in pattern to that of the mouse AA protein whose amino acid sequence analysis has been published by Eriksen et. al. (1976). No explanation was provided for the cleavage of the AA-protein into a comigrating doublet. It is quite likely that the doublet in AHCA, AZCA and mouse AA represents subunits of a single amyloid polypeptide denatured due to urea and/or SDS treatment.

The molecular weights of the AHCA and AZCA (murine AA produced in our laboratory), as determined by SDS-PAGE, were between 8.7 and 9.9 kd. However, by applying Hoy et. al's method (1974) which is based on amino acid composition of pure proteins, the calculated molecular weight of the AHCA was 8.018 kd (Table 2.2). These figures were significantly higher than that of the senile mouse amyloid (Matsumura et. al. 1982) but quite similar to mouse AA-amyloids reported previously (Eriksen et. al. 1976; Scheinberg et. al. 1976a). The isoelectric point of AHCA (5.1 to 5.3) was slightly lower than that of AZCA (pI 5.8). The pI values of human AA protein have been shown to range between 5.1-5.9 and 4.7-6.9. However, the amyloid sample preparation tested was not pure, the stained gel showed more than 17 bands (Westermark, 1982; Falk and Westermark, 1983).

The results of the Ouchterlony test (Fig. 2.9) and immunohistochemical staining (Fig. 2.8) of AHCA and AZCA using antisera raised against AHCA or AZCA showed strong cross-
reactivity between these two proteins. Adsorption of the antisera against heterologous amyloid proteins eliminated, with some exception, the antibody activity of the adsorbed sera when tested either by IFA or IP assays against homologous amyloid deposits. The RAHCA serum preadsorbed with lyophilized AZCA retained residual IFA reactivity against AHCA deposits. Furthermore, the single precipitation band showing a reaction of identity between AHCA, AZCA and SAA (azocasein-induced) against RAZCA serum (Fig. 2.9) in combination with results obtained by immunoblotting studies (Fig. 2.6) clearly indicates that AHCA is antigenically similar to AZCA and thus indeed an AA-type of amyloid. Similar to the predisposing factors required for the induction of AA-type of amyloidosis, hydatid-mice show chronic inflammation during the entire course of infection (Ali-Khan and Siboo 1980, Treves and Ali-Khan 1984a, b).

Despite antigenic and various physicochemical similarities between AHCA and AZCA the former retained its congophilic properties following KMnO₄ and trypsin treatment. Unlike AHCA, AA-fibrillar deposits in tissues are highly susceptible to the above treatment (Wright et al. 1974). Although retention of congophilic properties following KMnO₄ treatment is associated with AL-amyloid (immunoglobulin associated), neither anti-mouse IgG serum binds to AHCA deposits in tissues nor does RAHCA serum react against normal mouse serum by the immunodiffusion test (Fig. 2.9). To confirm whether the AHCA resistance to KMnO₄ and trypsin-treatment is a host strain-related property (C57BL/6J) or extends to other mouse strains as well, we tested AHC-induced
amyloid deposits in Balb/c, CBA and A/J mice. These samples responded similarly to KMnO₄ treatment as that of the C57BL/6J strain. The precise mechanism by which KMnO₄ treatment abolishes the congophilic property of AA-amyloid is obscure. Wright et al. (1974) have proposed that amyloid proteins with high arginine and/or lysine residues would be KMnO₄ and trypsin sensitive while human AL-type and some of mouse AA-type, which have relatively lower amounts of these two amino acid residues, would be resistant to the KMnO₄ and trypsin treatments. However, contrary to this explanation, our data on the amino acid composition show that AHCA contains relatively higher numbers of arginine and lysine residues/molecule of amyloid as compared to the murine AA amyloid (Table 2.3, Column E). A comparison of the amino acid composition between various murine amyloid proteins shows some similarities but a number of major differences (Table 2.3). For instance tryptophan was detected in trace amounts in the AHCA but the azocasein- and C. albicans-induced protein AA contained a substantial amount of this amino acid. Furthermore, glucosamine, galactosamine and trace amounts of methylated amino acids and ninhydrin position materials were also detected in the AHCA (Table 2.2). Because of low molar ratios of these materials, we suspect that they were constituents of AHCA.

In conclusion, it is believed that the AHCA, despite its anomalous response to KMnO₄ treatment and difference in amino acid composition (Table 2.3) shares common antigenic and physicochemical properties with murine protein AA. Furthermore, the AHCA protein is partially soluble in water and acid-urea solution is required to further solubilize the residual amyloid.
substance from the tissue. Whether the water and acid-urea soluble amyloid moieties are biochemically similar or different is not known.
CHAPTER THREE

CHARACTERIZATION OF THE PHLOGISTIC, CHEMOTACTIC, CYTOTOXIC AND AMYLOIDOGENIC POTENTIALS OF ALVEOLAR HYDATID CYST ANTIGENS OF ECHINOCOCCUS MULTILOCULARIS.
1. **INTRODUCTION.**

The migration or accumulation of cells of the immune system in inflamed or injured sites is a pathological process targeted at the removal of foreign antigens and products of autolysis (Leid and Williams, 1979). Macrophages, neutrophils, and eosinophils are the predominant cell types encountered in this process (Stevenson et al. 1981, Tanaka and Torisu 1978, Kay 1970). Parasite derived chemotactic and/or phlogistic components that cause intense inflammation in areas around the tissue-stage of parasites are well documented in the literature. *Entamoeba, Ascaris, Schistosoma* and *Echinococcus* species are among the many parasites that are known to produce some of the most active phlogistic and chemotactic components in host tissues (Tanaka et al. 1979, Nash et al. 1981, Ali-Khan and Siboo 1980, Chadee and Meerovitch 1984). Camp and Leid (1982) attributed the cellular influx against metazoan tissue parasites to parasite-elaborated chemotactic and phlogistic factors. In echinococcosis, an intense inflammatory response is associated with the histogenesis of alveolar hydatid cyst (AHC), and recurrent inflammation characterizes the course of AHD (Rausch 1954, Rausch and Schiller 1956, Ohbayashi et al. 1971). Introduction of AHCs, either subcutaneously or intraperitoneally in mice, triggers a prompt and intense inflammatory response (Ali-Khan and Siboo 1980; Devouge and Ali-Khan 1983; Treves and Ali-Khan 1984a). The influx
of cells is progressive and persists throughout the course of infection.

The examination of histologic preparations from the AHC at various time periods post-infection revealed that the inflammatory cells infiltrate both the stroma of the AHC and the surrounding host tissue. These cells bind to the surface of the AHC and cause degenerative changes in the laminated and germinal layers (Ali-Khan and Siboo 1980a). The predominant cell types that are involved in the inflammatory response in C57BL/6J mice are neutrophils and macrophages; eosinophils predominate at the focus of infection in Balb/c mice (Ali-Khan et al. 1983). The precise mechanism for the prompt cellular influx into the focus of infection in AHC-infected mice is not known. A number of factors such as AHC-derived factors, release of mediators and cytotoxic factors from degenerating inflammatory cells or local generation of immune complexes (ICs) and split products of complement have been suggested to be the effector components (Treves and Ali-Khan 1984b).

In AHD, despite the persistence of an intense inflammatory response during the entire course of infection, the AHCs progressively increase in size and destroy the surrounding host tissues. Several mechanisms have been postulated to explain the ability of the parasite to overcome the host immune system. Rausch (1954) attributed cell necrosis and tissue damage in AHC-infected gerbils to parasite elaborated toxic substances. Allan et al. (1981), using the unilocular hydatid cyst (E. granulosus) mouse model, observed a reduction in the number of thy-1 cells.
during the course of infection. They have suggested that T-cell depletion, which may be due to an "autoimmune destruction of parasite-reactive T-cells", is an important factor in the pathogenesis of the disease. Their observation strengthens the findings of Baron and Tanner (1977) who have shown that combined thymectomy and anti-lymphocyte serum treatment increased metastatic dissemination and significantly increased the weight of the AHC in mice. These observations suggest that T-cells aid in the control of the proliferation and growth of AHC in mice.

Ali-Khan (1978a, b) had hypothesized that the slow initial growth of the AHC reflected the hosts intact and functional control mechanisms. If the AHC established itself irreversibly (during this period) its subsequent growth combined with incessant flooding of the hosts lymphoid system either by circulating antigens and/or ICs might depress the hosts effector immune mechanisms. These predictions were subsequently supported by experimental evidence in AHC-infected mice. It was shown that the progressive growth phase of the AHC in mice corresponded with the generation of circulating ICs and their sequestration in the lymphoid organs (Ali-Khan and Siboo 1980b, 1983), thymus involution, disorganization of lymphoid tissue, depletion of T-cells and suppression of cell mediated immunity (Ali-Khan 1978a, b; Ali-Khan et al 1983; Devouge and Ali-Khan 1983). Further studies on the AHC-mouse model have suggested that numerical reduction in the intra-AHC leukocyte populations and IC-mediated blockage of Fc and C3b receptors of the inflammatory cells might further diminish the ability of intra-AHC leukocytes to bind and
cytolyse the opsonized cysts (Ali-Khan and Siboo 1981; Ali-Khan et al. 1983; Treves and Ali-Khan 1984a, b). Immune complexes have been shown to severely impair Fc and C3b receptors function of leukocytes and significantly modulate or in some cases abrogate cell-mediated cytotoxicity against target cells or metazoan parasites (WHO 1977; Karavodin and Ash 1982; Esparza et al. 1983). Despite the fact that AHCs induce severe immunopathological disorders in the host, the biological functions of AHC-antigens in the pathogenesis of AHD is not known. Annen et al. (1981) suggested that *E. multilocularis* might possess cytotoxic antigens similar to those found in the cyst fluid of *E. granulosus*. Such antigens, if they exist, can explain thymus involution, the depletion of T-cells and the observed degenerative changes around the parasite in AHC-infected mice (Ali-Khan 1978a, b; Ali-Khan et al. 1983). They would also explain the prolonged persistence and survival of the parasite in an immunologically competent host. No direct evidence has yet been presented for the possible existence of such antigens in AHCs. On the other hand, Treves and Ali-Khan (1984a, b) suggested that failure of the cell mediated immunity (CMI) and/or the antibody dependent cell cytotoxicity mechanisms contribute to the inability of the host to control the infection.

During the past few decades, interest has been rising towards the elucidation of the mechanisms which parasites, with potent chemoattractants, develop in order to evade the host immune system. It is not beneficial to the parasite to have "antigens" that will attract and alert the immune system of the
host to its presence, unless it is equipped with special defence mechanisms allowing it to survive by evading the host's defence mechanisms. Some of these special parasite defence mechanisms include antigenic variation or release of soluble antigens (ICs) in the host environment which will activate the generation of suppressor T-cells and induction of T- and/or B-cell tolerance causing immunodepression towards specific or unrelated antigens (Cohen 1976; Butterworth 1977; Faubert 1976, 1977). Coating of the parasite body surface with host proteins, which results in the alteration of the spacing of the accessible parasite antigens on the surface membrane (Philips-Quagliata et al. 1971), anatomical seclusion (intracellular location and cyst formation) (Purkerson and Despomier 1974; Remington and Krahenbuhl 1976; Siebert et al. 1981), proteolytic cleavage of specific immunoglobulins bound to accessible surface antigens (Auriault et al. 1981; Eisen and Tallian 1977), and non-specific binding of host immunoglobulins through the Fc fragment to the body surface of the parasite (Bogucki and Seed 1978; Auriault et al. 1981) have also been identified as evasive mechanisms which protozoan and metazoan parasites have developed through the course of evolution.

In certain diseases such as rheumatoid arthritis, tuberculosis, schistosomiasis and hydatidosis, severe chronic inflammation characterize the course of infection. Since chronic inflammation has been implicated in the pathogenesis of secondary amyloidosis (Janigan and Druet 1966), it becomes necessary to identify the nature of ABC-antigens which induce inflammation and
amyloidosis in AHD. In this chapter, alveolar hydatid cyst antigens were isolated and partially purified by affinity chromatography and gel filtration techniques. They were subsequently assayed for chemotactic, phlogistic, cytotoxic and amyloidogenic potentials.

2. MATERIALS AND METHODS.

a. Mice.

Six to eight week old C57Bl/6J or Balb/c mice were purchased from Jackson Laboratory, Bar Harbour, Maine, USA. They were used to maintain the parasite in the laboratory and to test the biological activities of E. multilocularis antigens extracted from purified preparations of AHC.

b. Preparation of the parasite antigens.

The methods of infection and recovery of cysts from dissociated AHCs have already been described in chapter-1. Cysts were washed extensively in ice-cold saline (0.85%) until the supernatant was clear and free of inflammatory cells. The sedimented cysts (50 ml packed volume) were collected in 200 ml of saline, examined microscopically for purity, and lysed by freezing and thawing. The lysed cysts were further homogenized and sonicated using a polytron probe (Brinkman Instruments, Rexdale, Ont. Canada) at cycle 8 for 5 minutes in an ice-water bath. The cyst suspension was then centrifuged at 20,000 x g for
1 hr and the supernatant was collected and dispensed in 10 ml aliquots and stored at -20°C.

c. Purification of AHC-antigen by affinity chromatography.

The cyst extract (AHC-antigen) was adsorbed extensively (at least 5 times) against rabbit anti-mouse immunoglobulins and rabbit anti-mouse IgG coupled to Cyanogen bromide (CNBr), activated Sepharose 4B (10 mg/g) following the manufacturers suggested coupling procedure (Pharmacia Fine Chemicals, Dorval, Qué. Canada). Purity of the adsorbed antigen was assessed by the gel diffusion test (Garvey et al., 1977). The adsorbed antigen did not show any precipitin bands when tested against rabbit anti-mouse serum. The purified antigen was then passed through a millipore filter (pore size = 0.22 μm) and stored at -20°C.

d. Quantification of cellular infiltrates following antigen administration in vivo.

Intradermal (ID) and intraperitoneal (IP) assays were used to test for the phlogistic activity of the parasite antigen. Essentially, the hair on the back of the mice (1 cm below the neck and 1 cm to the right of the midline across the back of the animal) was clipped and 0.05 ml of the antigen containing 35 or 70 μg of protein was injected into the dermis using a 27-gauge needle. The same mouse received 0.05 ml of saline or 1% bovine serum albumin (BSA) 2-3 cm below the antigen injection site for control purposes (Kay, 1970). After 3, 6, 12, 24, and 48 hr following the administration of the antigen, the injection sites (1 cm in diameter) were excised and immediately fixed in Hollands...
fixative. The skin samples were trimmed, embedded in wax, sectioned at 5 μm and stained with the Dominici stain as modified by Litt (1963). From each section, a total of 20 random high-power fields were examined and the inflammatory cells counted. Morphologic and histochemical criteria were used to identify neutrophils, monocytoid cells, mast cells, eosinophils, and lymphocytes.

The phlogistic activity of the affinity purified AHC-antigens were also tested IP. In this assay 1 ml of the antigen containing 200 μg of protein was injected IP in mice. After 12 and 96 hr, the cells from the peritoneal cavity were collected in RPMI-1640, counted and cytocentrifuged on glass slides. The cells were stained with the Dominici and Giemsa stains after fixation in absolute methyl alcohol. For control purposes, 1 ml of 10% azocasein in bicarbonate buffer or 1 ml of saline was also injected IP into mice. The cells were collected and processed for light microscopy as described for the experimental group.

e. Purification of AHC-antigens by gel filtration.

The AHC-antigen was fractionated through a Sephacryl S-300 column (1.5 x 90 cm) equilibrated with 0.1 M borate buffered saline (pH 8.2). The eluent fractions were monitored at 280 nm. Individual peaks were collected and concentrated under nitrogen using the Amicon cell fitted with a PM 10 membrane (MW cut off 10,000). Total protein and carbohydrate contents of the antigen were determined using the Bio Rad protein assay kit (as described in chapter 2) and the colorimetric method of Dubios et al. (1956)
respectively. The fractions were then dialysed against RPMI-1640 using spectrapore dialysis membrane number 3 (MW cut off 3,500). Peaks 1 and 2 contained sufficient amounts of protein and carbohydrate to allow the chemotaxis assay to be performed.


Mice were injected IP with 2 ml of 7.5% aqueous azocasein and sacrificed by cervical dislocation after 12 hr for the isolation of neutrophils or after 96 hr for the isolation of macrophages. The peritoneal cavities were washed with RPMI-1640 supplemented with 20 mM Hepes and 10% fetal calf serum (FCS), pH 7.2. The washings from several mice were pooled and centrifuged at 300 x g for 15 minutes at 4°C. The cells were washed 2 times and then resuspended to contain 3 x 10^6 viable neutrophils or 2 x 10^6 viable macrophages per 1 ml of RPMI-1640.

g. The_chemotaxis_assay.

The chemotaxis assay was performed as has been described by Snyderman et al. (1974) and Stevenson et al. (1981). Briefly, several dilutions of the antigen containing 1, 10, 50, 100 and 200 ug of protein were made in RPMI-1640 supplemented with 20 mM Hepes and injected into the lower compartment of Boyden chambers (Neuroprobe Corp. Calif. USA). Polycarbonate membranes of 3 or 5 um pore size were used for the migration of neutrophils or macrophages, respectively. One milliliter of the cell suspension containing 3 x 10^6 neutrophils or 1 x 10^6 macrophages was added to the upper chamber. The chambers were then incubated in a humidified incubator at 37°C for 3 hr. In
order to ascertain random migration of cells across the membrane, a similar set up was prepared either without the antigen in the lower chamber or with an equivalent amount of the antigen in the upper chamber. To test for positive migration of cells across the membrane, endotoxin-activated mouse serum (EAMS) was used at 1:100 dilution. The method of Stevenson et al. (1981) was followed for the preparation of EAMS. At the end of the incubation period, the cell suspension was removed from the upper chamber with a cotton swab. The filters were then fixed in absolute methyl alcohol and stained with 0.3% aqueous toluidine blue solution for 1 minute, destained in phosphate buffer, pH 6.7, air-dried overnight and mounted on glass slides with permount. The chemotactic response was expressed as the mean total number of migratory neutrophils or macrophages per oil immersion field (OIF) ± standard error of the mean for quadruplicate filters.

h. Assay for amyloidogenic activity of AHC-antigen.

In order to test for amyloidogenic activity of the AHC-antigen, 4 groups of mice were inoculated IP with varying concentrations of the antigen at 24 hr intervals as indicated in table 3.1. The mice were sacrificed 24 hr after the last inoculum. Amyloid deposits in the spleens were assessed by polarization microscopy after staining with Congo red as has been described in chapter 1.

i. Assays for cytotoxicity and blastogenic response.

Spleen cells from 10 week old C57BL/6J mice, were
prepared as described by Ali-Khan et al. (1983). Briefly, $2 \times 10^5$ viable spleen cells were suspended in 100 μl of culture medium (RPMI-1640 containing 10 mM Hepes buffer, 2 mM L-glutamine, 5% FCS, 100 units of penicillin G and 100 μg of streptomycin sulphate /ml and $2 \times 10^{-5}$ M mercaptoethanol, final pH 7.3) and cultured in sterile round-bottom microtitre plates. One hundred microliters of sterile AHC-antigen, crude or purified, containing 10, 50, 100 or 200 μg of protein was added in triplicate to different wells. The control wells received 100 μl of the culture medium or normal mouse serum. The final volume in each well was 200 μl. In some of the control wells, 100 μl of concanavalin A (Con A, 0.3 μg/ml) or lipopolysaccharide (LPS, 10 or 15 μg/ml) was added. The plates were then incubated at 37°C in a humidified chamber (5% CO₂ in air). The viability of the cells after 1, 5, 9, 24, 48 and 72 hr of incubation was assessed by the trypan blue exclusion method (Garvey et al. 1977). Blastogenic response was assessed using radio-labelled thymidine. Ten microliters (1 μCi) of tritiated thymidine [({methyl-}³H) thymidine] (New England Nuclear, NEN, Que. Canada, NET-027Z), specific activity 78.6 Ci/mMol., in phosphate buffered saline (PBS) was added to each well. The cells were harvested after 4 hr with an automatic cell harvester (Flow Laboratories, Ont. Canada), and the uptake of tritiated thymidine by living cells was measured in a Beckman liquid scintillation (LS) counter (8000 series). The results are presented as the mean counts per minute (CPM) of triplicate cultures.
a. Time-course of cellular infiltration into skin.

The crude AHC-antigen contained 3 mg of protein and 1 mg of carbohydrate/ml. The affinity-purified AHC-antigen which was used in this study contained 700 ug of protein and 250 ug of carbohydrate/ml. The injection of antigen into the dermal layer of the skin induced significant accumulation of neutrophils in the tissue. The kinetics of this response did not change significantly when the antigen concentration was increased from 35 to 70 ug of protein (Fig. 3.1 a,b). In general, no tissue eosinophilia was detected at the injection sites. In contrast, neutrophils were present in significantly high numbers at 3, 6 and 12 hr after the injection. The peak response was observed as early as 6 hr and by 24 to 48 hr the number of the extravasated neutrophils reached the level of the control mice which were injected with normal saline. The neutrophils showed a single peak during the time-course response and the peak response appeared to be time-dependent. The peak response however, was significantly higher when 70 ug of the antigen protein was injected into the skin (Fig. 3.1b). The neutrophils appeared in significant numbers at 3 hr inside and outside the hyperemic blood vessels above the thin muscular layer of the dermis (Paniculus Carinosus) (Fig. 3.2c). By 6-12 hr, these cells had infiltrated the fibrinous edematous loose connective tissue of the reticular layer of the dermis (Fig. 3.2d). At 12 hr, the migration process from the blood
Figure 3.1

Time course of intradermal accumulation of neutrophils (closed circle), mononuclear leukocytes (open circle) and eosinophils (closed square) after intradermal injection of: (A) 0.05 ml of AHC-antigen containing 35 ug of protein (solid line), (B) 0.05 ml of AHC-antigen containing 70 ug of protein (solid line), or (C) 0.05 ml of 1% bovine serum albumin (solid line) in normal C57BL/6J mice. The broken lines represent responses to an injection of 0.05 ml of 0.85% saline (control). The data are based on the mean ± standard error of the mean for each cell type in four mice.
Figure 3.2

Typical pictures of cellular infiltration and pathological changes in the skin of C57BL/6J mice at 6 hours following the intradermal injection of 0.05 ml of 0.85% saline (A) or 0.05 ml of AHC-antigen (70 ug of protein) (B, C, & D).

(A) Skin section of a control mouse. X 100.

(B) Skin section of AHC-antigen injected mouse. Note the intense inflammatory response (neutrophilic infiltration) in the panniculus carnosus (pc) and reticular layer (rl) of the dermis. X 100.

(C) Accumulation of neutrophils and migration through a blood vessel in the rl of the dermis. X 1000.

(D) Neutrophil accumulation, edema and fibrin formation at the junction of the pc and rl of the skin. Note the low numbers of mononuclear leukocytes and the absence of eosinophilic granulocytes. X 400.
vessels appeared to have subsided and between 24 to 48 hr only a few extravasated neutrophils were present at the test sites. The saline injected skin sites showed occasional neutrophils, eosinophils or monocytoid cell infiltrates (Fig. 3.2a). The monocytoid cell response at the antigen-injected skin site was very low. No significant increase in the number of monocytoid cells was observed at any time periods except at 24 hr following the injection of 35 ug of AHC-antigen protein. In contrast, the inflammatory response to BSA differed both qualitatively and quantitatively from that of the AHC-antigen. First, the neutrophils were very low in number and second, the peak neutrophilic response was absent (Fig. 3.1c). The monocytoid cells were however, significantly higher in number and peaked at 24 hr. The peak response of the monocytoid cells was 3 times higher than that of the antigen-induced response.

b. The time course of cellular infiltration into the peritoneal cavity.

The total number of cells that infiltrated the peritoneum of mice either at 12 or 96 hr after AHC-antigen injection was not significantly different (Fig. 3.3 a, b). However, at 12 hours, 90% of the cells consisted of neutrophils(Fig. 3.3a), and at 96 hours 70% of the cells were monocytoid cells (Fig. 3.3b). In both instances, a slight increase in the number of lymphocytes and eosinophils was measured when compared to the saline-injected control group. In the azocasein-injected mice, most of the cells at 12 hr were neutrophils and at 96 hr, monocytoid cells. No
Figure 3.3
Total and differential leukocyte counts of intraperitoneal infiltrates in normal mice injected with 1 ml of (A) 0.85% saline, (B) AHC-antigen (200 ug of protein), or (C) 10% azocasein solution. Mononuclear leukocytes (open bars), eosinophils (dotted bars), neutrophils (striped bars), and lymphocytes (black bars) after 12 hours (1) or 96 hours (2) post-injection.
significant increase in the number of eosinophils or lymphocytes was observed either at 12 or 96 hr over the control group.

c. In vitro chemotaxis.

The data on in vitro chemotactic activity of the AHC-antigen in Boyden chambers are shown in figure (3.4 a, b). The antigen was chemotactic for both neutrophils and monocytoid cells and the responses were dose-dependent. All of the antigen concentrations tested induced chemotaxis at levels above the random migration except at a concentration of 1 ug. The peak migratory response of neutrophils or monocytoid cells was observed when 100 ug protein of AHC-antigen was added to the lower chamber. In both instances it was comparable to the chemotactic activity of EAMS at a dilution of 1:100. On the other hand, when the same concentration of the antigen was added to the cells in the upper compartment of the chamber, very few neutrophils or monocytoid cells migrated to the lower surface of the membrane. The number of migrant cells was not significantly different from the control group where no chemotactant was added. The kinetics of the monocytoid cell response was similar to that of the neutrophils. However, the number of migratory monocytoid cells was approximately 50% of the neutrophils at all time periods including the peak response (Fig. 3.4 a, b).

d. Chemotactic activity of purified AHC-antigen.

The elution pattern of AHC-antigen on Sephacryl S-300 and the in vitro chemotactic activity of peaks 1 and 2 are shown in figure (3.5). Peaks 1 and 2 showed intense chemotactic activity.
Kinetics of chemotactic responses of azocasein-induced peritoneal neutrophils (A) or macrophages (B) in response to EAMS, saline and various concentrations of AHC-antigen in Boyden chambers. The data are presented as the mean total neutrophil or macrophage count per oil immersion field ± the standard error of the mean for quadruplicate filters.

Figure 3.4
Figure A: PMNs/OIL IMMERSION FIELD

Figure B: MACROPHAGE/OIL IMMERSION FIELD

CHEMOATTRACTANT, µg protein.

Graphs showing the response of PMNs and macrophages to different concentrations of chemotactants.
The kinetics of the cellular response to both these fractions was similar to that of the crude hydatid antigen. However, the chemotactic potential of peak 1 was significantly higher than peak 2.

e. Amyloidogenic potential of AHC-antigen.

Table 3.1 shows the correlation between amyloid deposition and the effect of IP inoculation of AHC-antigen in mice. One milligram of the antigen protein given in 2 inocula 24 hr apart, induced amyloid deposition. Repeated IP administration of the antigen in mice induced a slight but not significant increase in the amount of amyloid deposits in the spleens and livers. When a single inoculum of 2 mg of the antigen was given IP, amyloid appeared in the spleens but not the livers after 48 hr. The amount of amyloid deposited in response to the single antigen injection was not significantly different from the mice which had received the same amount of antigen in 4 separate injections (Table 3.1, group A-2).

f. Cytotoxicity of hydatid antigen.

Figure 3.6 presents the data of the cytotoxicity assay. At 72 hr, approximately 30% of the unstimulated splenocytes were found dead. This level was not significantly different from that of LPS- or Con A-stimulated cells. The observed decline in viability is normal. It is known to occur in primary spleen cell cultures (Adler et. al. 1970; Annen et. al. 1981). However, when the cells were incubated with the antigen a much higher mortality rate was observed (Fig. 3.6). The exposure of cells to varying
Figure 3.6

Ascending chromatogram of AHC-antigen on Sephacryl S-300 column and the chemotactic response of azocasein-elicited neutrophils (PMN) to 100 ug of protein (black bar) or 50 ug of protein (white bar) of 1 and 2. The random migration level and the chemotactic response to EAMS are presented by x and y, respectively. The data are presented as the mean of quadruplicate filters ± the standard deviation. The elution of the molecular weight markers is shown in the figure (from left to right: dextran blue, mouse IgG and myoglobin type 1 from equine skeletal muscle).
ABSORBANCE AT 280 n.m.

FRACTION NUMBER

0.2
0.1
0.05
0.0

0 10 20 30 40 50 60 70 80 90

2000 150 18 Kd

NUMBER OF PMNS/0.1 F.

60
50
40
30
20
10
0
Table 3.1

Effect of alveolar hydatid cyst antigens on the induction of amyloid deposition in C57BL/6J mice.

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Amount of AH antigen</th>
<th>No. of mice</th>
<th>No. of injections</th>
<th>% cross-sectional area covered with amyloid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Spleen</td>
</tr>
<tr>
<td>A1</td>
<td>500</td>
<td>5</td>
<td>2</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>A2</td>
<td>500</td>
<td>5</td>
<td>4</td>
<td>9.3 ± 1.7</td>
</tr>
<tr>
<td>A3</td>
<td>500</td>
<td>6</td>
<td>6</td>
<td>12.2 ± 1.6</td>
</tr>
<tr>
<td>B1</td>
<td>100</td>
<td>5</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>B2</td>
<td>100</td>
<td>5</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>100</td>
<td>5</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>C2</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>10</td>
<td>5</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>2000</td>
<td>4</td>
<td>1</td>
<td>10.3 ± 1.7</td>
</tr>
</tbody>
</table>

1. ug of protein.
2. Number of antigen injections at daily intervals.
concentrations of AHC-antigen showed that the 200 ug dosage caused a marked decrease in the viability of splenocytes at all time periods except after 1 hr. The decline in viability was sharp during the first 5 hr in culture and gradual during the reminder of the incubation time. After 5 hr, 65% of the cells cocultured with the antigen were dead. The maximum mortality rate recorded was 90% after 24 hr in culture.

g. Mitogenic effect of AHC-antigen.

The incorporation of tritiated thymidine by Con A, LPS, or AHC-antigen stimulated cells as well as the unstimulated cells is shown in figure 3.7. It is clear that the unstimulated (control) cells incorporated minute amounts of radioactivity, while the Con A or LPS stimulated cells incorporated much higher amounts. In contrast, splenocytes cultured in the presence of the antigen (50, 100 or 200 ug for 24 hr) did not incorporate any significant amount of tritiated thymidine. The level of incorporation was comparable to the background level although it was much lower than the unstimulated control group.
The viability of Blouse spleen cells as assessed by the trypan blue exclusion test at various time periods following exposure to various concentrations of AHC-antigen 200 ug of protein (black circles), 100 ug of protein (black squares), 50 ug of protein (black triangles), RPMI-1640 (broken line), 4 ug of LPS (white squares) or 0.3 ug of Con A (white triangles). The data are presented as the mean counts of triplicate cultures ± the standard error of the mean.

The incorporation of tritiated thymidine by murine spleen cells at 24, 48 and 72 hours following exposure to AHC-antigen at concentrations of 200 ug of protein (white circles), 100 ug of protein (white triangles) and 50 ug of protein (white squares) or to mitogenic stimulation by 4 ug of LPS (black squares) or 0.3 ug of Con A (black circles). The levels of incorporation of untreated spleen cells (control) is represented by the broken line. The data are presented as the mean of triplicate cultures ± the standard error of the mean.
4. DISCUSSION.

The data obtained clearly implicate the presence of potent phlogistic, chemotactic, cytotoxic and amyloidogenic components in the AHC-antigen. The parasite antigen used, especially in vivo assays, was affinity purified and was devoid of any host serum components. Therefore, the observed biological activities of AHC-antigens can be safely associated with the AHC-antigens. When the antigen was chromatographed on Sephacryl S-300 it eluted into four distinct protein peaks. The first and second fractions were chemotactic, indicating that there are at least two potent chemotactic factors in the antigen preparation. The molecular weight of the first ranged between 1000-2000 Kd, and the second between 30-100 Kd (Fig. 3.5).

The parasite antigens showed considerable amount of phlogistic activity in vivo. Large numbers of inflammatory cells were observed in the skin sections and in the peritonea of mice following the administration of the antigen. It is important to note that eosinophilia was not a major feature of the inflammatory process. In fact, very few eosinophils were present at either site. This observation is important for 2 reasons. First, in hydatidosis, eosinophilia is not a significant feature of the disease process as in the case of other tissue helminthias which parasitize mammals (Torisu et. al. 1975; Tanaka and Torisu 1978; Tanka et.al. 1972; Ali-Khan and Siboo 1980). Second, it has
been shown that immune complexes, lymphokines and host serum proteins are potent eosinophil chemotactic factors (Kay 1970; Ward 1971; Wissler et. al. 1972). Therefore, the failure of AHC-antigen to elicit an eosinophilic response clearly indicates the absence of such components from the antigen preparation. However, potent neutrophil chemotactic factors (NCF) were present in the antigen. Tanaka et. al. (1979) found 2 NCFs in the extract prepared from adult Ascaris. In contrast to the AHC-derived neutrophil chemotactic factor (AHC-NCF), Ascaris-NCFs were found to be heat labile and considerably less potent. One milligram of the purified Ascaris-NCF was required to induce a significant degree of chemotaxis in vitro. The Ascaris-NCFs were not affected by dialysis or lyophilization. More potent NCFs were identified in the extract of plerocercoids of Spirurometra erinacei (Horri et. al.1984). The approximate molecular weight of the first factor was in the range of 150 Kd and 10-20 Kd for the second. However, the potency of these NCFs appear to be lower than the AHC-NCF.

AHC-antigens were also amyloidogenic in nature. However, the precise mechanism of amyloid induction in the AHC-mouse is not entirely clear. Recent studies in this laboratory indicate that AHC infection in C57BL/6J and CBA mice stimulates a low level of SAA response (Sipe and Ali-Khan 1985, unpublished data). However, the profile of the SAA response in the AHC-mice is typical of chronic inflammation (McIntyre et. al.1982). The level of SAA concentrations in the sera of AHC-mice did not exceed 19 ug/ ml (Sipe and Ali-Khan 1985, unpublished data) which amounts
to approximately 2 to 4% of the SAA concentration observed during acute episodes in mice stimulated with azocasein or lipopolysaccharides (McAdam and Sipe 1976; Tatsuta et al. 1983; Kisilevsky 1983). Recent developments in the study of AA-amyloidosis have led to the identification of SAA as the possible serum precursor of tissue amyloid protein AA (Levin et al. 1973; Benditt et al. 1979; Hoffman et al. 1984). It has been shown that inflammatory stimuli induce macrophages to secrete Interleukin 1 which in turn stimulates hepatocytes to synthesize and secrete SAA (Sztein et al. 1981; Hoffman and Benditt 1982; McAdam et al. 1982; Sipe et al. 1982). However, the identification of pathogenetic factors which induce the conversion of SAA into fibrillar amyloid remains an urgent and challenging problem in amyloidosis. Kisilevsky (1983) has proposed that impairment of macrophage function and a concomitant appearance of amyloid enhancing factor (AEF) in the tissues are crucial factors in the pathogenesis of amyloidosis. Neither the nature nor the etiology of macrophage impairment is known. Kisilevsky and Axelrad (1976) suggested that the impairment of macrophage function was due to one of the following aspects: (A) change in permeability or selectivity, resulting in the degradation of a normally inaccessible substances, (B) quantitative increase in the processing activity, resulting in the accumulation of the amyloid substance which otherwise would be present at undetectable levels, and (C) alteration in the degradative pathway resulting in the accumulation of large amounts of intermediates which are the amyloid subunits. These intermediates would normally be.
cleared from the intracellular as well as extracellular locations. Most amyloidogens used in the experimental induction of secondary amyloidosis in laboratory animals (casein, azocasein, bacterial lipopolysaccharide, and silver nitrate) have been shown to induce SAA response and the generation of AEF (Levin et al. 1973; Kisilevsky and Axelrad 1976; Hardt and Ranlov 1976; Benditt et al. 1979; Axelrad et al. 1982; Hoffman et al. 1984). Similar to these amyloidogens, AHC-infection induces SAA synthesis and the formation of an AEF-like factor (see chapter 4) in the spleens and livers of AHC-mice. The AHC-induced AEF is functionally identical to those induced by azocasein and silver nitrate (see table 4.3). These findings firmly establishes the role of the parasite in the induction of secondary amyloidosis.

In this chapter, experimental evidence has been provided to demonstrate the cytotoxic effect of AHC-antigen on murine spleen cells. Eighty to 90% killing was observed with either the affinity purified crude antigen or fraction 1 of the AHC-antigen. The loss of cell viability was further confirmed by the residual levels of thymidine incorporation by spleenocytes. The cytotoxic antigens are most probably somatic in origin. They are released either locally or in the hosts circulation as a result of degenerative changes in the AHC (Ali-Khan and Siboo 1983). The cytotoxic nature of the AHC-antigen is of considerable interest and may be helpful in explaining the induction of amyloidosis in the AHC-mice.

The dysfunction of hosts immune mechanisms has been
implicated in the pathogenesis of secondary amyloidosis in the casein mouse model. Involution of the splenic white pulp and thymus, T-cell depletion, poor reactivity of T-cells to mitogens and impairment of macrophage function were noted in mice given daily injections of casein, azocasein or nitrogen mustard (Graef et al. 1948; Telium 1964, 1968; Druet and Janigan 1966; Ranlov and Jensen 1966; Cohen and Claesson 1971; Cathcart 1972; Hardt and Ranlov 1972). It is significant to point out that all these observations occur in the AHC-mice along with intense inflammation, high cell mortality, and cell necrosis in and around the AHC (Rausch 1954; Webster and Cameron 1969; Ali-Khan 1978a, b; Ali-Khan and Siboo 1980a, b; Devouge and Ali-Khan 1983; Treves and Ali-Khan 1984a, b). The mechanism of action of E. multilocularis cytotoxin is not clearly understood. What is evident from the data is that the action of the cytotoxin is dose dependant and at least a minimum period of 5 hours is required to detect a significant degree of killing. The toxic effect is most probably directed at the metabolic processes of the cell. The chemotactic and cytotoxic activity of the AHC-antigen can therefore be considered as important factors by which the parasite attracts and kills the effector cells and ensures its prolonged survival in the host. The histopathological changes and the immunopathological disorders that accompany AHD support this hypothesis (Rausch 1954; Cameron and Webster 1969; Ali-Khan 1974, 1978; Vogel 1977; Ali-Khan and Siboo 1980; Annen et al. 1981; Treves and Ali-Khan 1984a, b).
CHAPTER FOUR

ISOLATION AND CHARACTERIZATION OF AN AMYLOID ENHANCING - LIKE FACTOR FROM MICE INFECTED WITH ECHINOCOCUS MULTilocularis.
1. INTRODUCTION

The transfer of viable or nonviable spleen cells from pre-amyloidotic or amyloidotic animals to isogeneic or allogeneic recipients has been shown to enhance amyloid formation following antigenic stimulation (Janigan and Druet 1968, Ranloviq 1968, Janigan et al. 1969, Axelrad et al. 1982). A substance referred to as the donor (transfer factor) or the amyloid enhancing factor (AEF) has been identified in spleen cell homogenates. Passive transfer of this factor has been shown to accelerate amyloid deposition in the recipient mice (Janigan 1968; Axelrad et al. 1982; Kisilevsky and Boudreau 1983). Several attempts have been made to characterize this crucial factor and the results compiled from the literature are summarized in Table 4.3. It is apparent, from the table, that no agreement has so far been reached as to the exact nature of the transfer or donor factor. Much of our current knowledge on AEF comes from the work done at Queen’s University by Drs. M.A. Axelrad and R. Kisilevsky. Amyloid enhancing factor is a high molecular weight glycoprotein (Axelrad et al. 1982). It appears to be present in detectable quantities in the ribosomes, microsomes and membrane fragments devoid of ribosomes (Axelrad et al. 1982). It appears in host spleens 24–48 hours prior to amyloid deposition. When transferred to isogeneic mice and followed by an inflammatory stimulus, amyloid formation in the spleens of the recipient mice occurs in less
than 48 hours. However, when the inflammatory stimulus is removed regression and complete dissolution of amyloid deposits were observed by day 30 (Kisilevsky and Boudreau 1983). Although AEF is associated with amyloidosis, it is not the amyloid A-protein, interleukin-1 or the amyloid P-component (Axelrad et al. 1982). Furthermore, the bioactivity of AEF is not affected by DNAase or RNAase but is abolished by protease or pronase treatments (Axelrad and Kisilevsky 1980, Axelrad et al. 1982).

All the information available in the literature on AEF or AEF-like factors such as the donor or transfer factor, originates from studies done on mice injected with an inflammatory substance (casein, azocasein, albumin, nitrogen mustard or silver nitrate). In this chapter, evidence is presented for the first time about the existence of an AEF-like factor from the tissues of a parasitized host. Some of the biological as well as physicochemical characteristics of the alveolar hydatid cyst induced AEF referred to as AHC-AEF are presented. The following aspects are emphasized:

(1) time of appearance
(2) biological half life
(3) dose response
(4) chemical nature
(5) partial purification by gel filtration
(6) effect on amyloid deposition in AHC-infected mice.
2. MATERIALS AND METHODS

a. Source of AEF.

Spleens from C57BL/6J mice infected intraperitoneally (i.p.) with 50 ± 10 cysts of Echinococcus multilocularis, were excised postmortem at weekly intervals post-infection (p.i.) They were either used immediately or stored at -20°C until used. Spleens from amyloidotic Balb/c or A/JAX mice, infected with a similar inoculum, were also used for the extraction of AHC-AEF.

b. Preparation of AHC-AEF.

The AHC-AEF was extracted with 10 ml borate-buffered saline (BBS), pH 7.4, per gram of wet weight of spleen. The spleens were homogenized in a Potter-Elvejhem homogenizer (30-40 strokes) followed by homogenization and sonication using a polytron probe (Brinkman Instruments, Rexdale, Ont. Canada) at cycle 8 for 5 minutes in an ice-water bath. The suspension was centrifuged at 30,000 x g for one hour at 4°C. The supernatant, designated as the crude AHC-AEF was collected and stored at -20°C in 10 ml aliquots in polypropylene tubes. Most of the data presented in this chapter were obtained from experiments using the crude AHC-AEF, unless otherwise indicated.

c. Route of administration of AHC-AEF.

The AHC-AEF was administered i.p. Immediately prior to the injection of the inflammatory stimulus. In one experiment the stimulus was given at various time periods after the administration of AHC-AEF. In all experiments, C57BL/6J mice
were used to test the bioactivity of AHC-ABF. A minimum of 6 mice per group were used in all of the experiments unless otherwise stated. Except for the dose response experiment, one milliliter of AHC-AEF, containing 10 mg of protein, was injected into mice in all of the experiments.

d. **Inflammatory stimulus.**

A single subcutaneous injection of 0.5 ml of a 2% silver nitrate solution in double distilled water (DDW) served as the inflammatory stimulus in most of the experiments. The surface area of spleen obliterated with amyloid was quantified after 48 hours. In one experiment, 50 ± 10 AHCs substituted the silver nitrate solution in order to study the effect of AHC-ARF on amyloid deposition in AHC-infected mice. The amount of amyloid deposited in the splenic tissues of these mice were quantified after 1, 2, and 3 weeks p.i.

e. **Purification of AHC-ABF by gel filtration.**

Crude AHC-AEF was applied to a Sephacryl S-300 gel column (1.0 x 90 cm) equilibrated with borate buffered saline, pH 7.4. The eluent fractions were monitored at 280 nm. Individual peaks were collected and dialysed against DDW in spectrapore dialysis membrane number 3 (MW cut off 3,500) and lyophilized. Each peak was reconstituted to the original volume (before fractionation) in BBS and tested for its bioactivity in comparison with the crude AHC-ABF.
f. Methods of amyloid quantification.

Mouse spleens were prepared for polarization microscopy as described in chapter 1. The surface area covered with amyloid in amyloidotic spleens was measured using computer assisted morphometry or as has been described in chapter 1.

g. Methods of enzymatic digestion.

One milliliter of the Crude AHC-AEF was incubated for 45 minutes with either of the following:

(1) 1 or 5 mg of deoxyribonuclease I and II (Sigma Chemical Company, MO. USA, D-47.63 and D-87.64); at 25°C pH 4.5-5.0.

(2) 1 or 5 mg of ribonuclease A (Sigma Chemical Company, MO. USA, R-51.25); at 37°C pH 7.0-7.5.

(3) 1 or 10 mg of trypsin (Sigma Chemical Company, MO. USA, T-80.03); at 25°C pH 7.0-7.5.

(4) 1 or 10 mg of pepsin (Sigma Chemical Company, MO. USA, P-68.87); at 37°C pH 2.0.

h. Methods of protein determination.

Soluble protein in AHC-AEF preparations was measured using the Biorad protein assay kit as described in Chapter 2.

i. Effect of temperature and pH on AHC-AEF.

Ten milliliters of AHC-AEF were incubated at 4°C for 10 days or in a boiling water bath for 60 minutes with frequent mixing. To measure the effect of pH, AHC-AEF was dialysed for 12 hours against (a) acetate buffer, pH 3.0 (b) borate or Tris buffer, pH 9.5 or (c) RPMI 1640, pH 7.4 overnight at 4°C, using spectrapore
dialysis membrane number 3 (MW cut off 3,500). Following dialysis the pH of AHC-AEF was adjusted to 7.0-7.5 and then tested in vivo.

j. Ultracentrifugation of AHC-AEF.

In order to determine whether the AHC-AEF activity is present in the sedimentable or soluble moieties of the spleen cell extract, 4 ml aliquots of crude AHC-AEF were ultracentrifuged at 250,000 x g for 2.5 hours or at 100,000 x g for 12 hr in a Beckman ultracentrifuge, model L7 80, using the SW-60 head rotor (Beckman Instruments, USA). The rotor temperature was adjusted not to exceed 10 C.

k. Effect of administration of various amounts of AHC-AEF on amyloid deposition in mice.

One tenth of a milliliter, 0.5, 1.0, 2.0 or 3.0 milliliters of AHC-AEF was injected i.p. in mice (4 mice / group) and followed by a single s.c. injection of silver nitrate. The mice were sacrificed after 48 hr and their spleens were excised and processed for polarization microscopy following Congo Red staining.

l. Determination of the half life of AHC-AEF in vivo.

Thirty five C57BL/6J mice were injected i.p. with 1 ml of AHC-AEF. At the following days 1, 3, 7, 14, 21 or 27 after the administration of AHC-AEF, 5 mice were injected s.c. with silver nitrate and sacrificed after 48 hr. The amount of amyloid deposits in the spleen were measured as has been described earlier. Five mice were injected with AHC-AEF and silver nitrate simultaneously as the control.
3. RESULTS

a. **Time of appearance of AHC-AEF activity.**

AHC-AEF activity was detected in the spleen homogenates of all the AHC-infected C57BL/6J mice examined at 5 weeks p.i. Spleen extracts prepared from AHC-infected Balb/c and A/JAX mice also demonstrated AHC-AEF activity. These spleens were obtained at 5 and 12 to 16 weeks p.i. respectively.

b. **Dose response.**

Figure 4.1 shows the effect of the administration of increasing amounts of AHC-AEF in C57BL/6J mice. The maximum amyloid deposition in the recipient spleens were obtained with 1 ml of the AHC-AEF; 15% of the surface area in the spleen was obliterated with amyloid deposits. The location and appearance of amyloid deposits were similar to that described for C57BL/6J mice in chapter 1. Lower or higher amounts (0.5 or 3 ml) of AHC-AEF dosage reduced significantly the amount of tissue amyloid.

c. **Effect of enzymatic treatment and refrigeration on AHC-AEF activity.**

Figure 4.2 shows the effect of enzyme-treatment and incubation at 4°C on the activity of AHC-AEF. Enzyme-treatment reduced the activity of AHC-AEF by approximately 25% when compared to the control group. However, no significant difference was detected between the various enzyme-treatments. The refrigeration of AHC-AEF at 4°C for 10 days before its
Effect of the inoculation of various amounts of AHC-AEF in C57BL/6J mice on amyloid deposition. Data are presented as the mean percent area of spleens covered with amyloid from 4 C57BL/6J mice after 48 hours of the administration of AHC-AEF (10 mg of protein/ ml) and challenge with silver nitrate.

Effect of refrigeration and enzymatic digestion on the bioactivity of AHC-AEF. Each histogram represents the mean percent area of spleens covered with amyloid from 4 C57BL/6J mice in response to 1 ml of (A) untreated AHC-AEF, (B) AHC-AEF incubated at 4°C for 10 days, (C) untreated AHC-AEF, (mice were sacrificed after 10 days following the administration of AHC-AEF and the inflammatory stimulus), (D) untreated AHC-AEF, (mice were sacrificed after 45 days following the administration of AHC-AEF and the inflammatory stimulus), (E) Trypsin-digested AHC-AEF, (F) Pepsin-digested AHC-AEF, (G) DNAase-digested AHC-AEF, and (H) RNAase-digested AHC-AEF. All mice were sacrificed after 48 hours of the administration of AHC-AEF and the inflammatory challenge (silver nitrate) except (C) and (D) which were sacrificed after 10 and 45 days post-treatment respectively.
administration did not reduce its activity. However, when spleens were examined at 10 or 45 days, instead of the regular 48 hour period, following the administration of AHC-AEF plus the inflammatory stimulus a 50% and 130% increase in the amount of amyloid deposited was recorded respectively (Fig. 4.2).

d. Effect of boiling and pH on AHC-AEF activity.

Table 4.1 shows the effect of low or high pH and boiling on the activity of AHC-AEF. Either low or high pH treatment, as well as boiling, completely abolished the bioactivity of AHC-AEF.

e. Partial purification and bioactivity of AHC-AEF fractions.

Figure 4.3 shows the chromatographic profile of borate buffer extracted AHC-AEF on a Sephacryl S-300 gel column. Four major protein peaks were eluted. The bioactivity of each peak is presented in table 4.1. Peak-2 had the highest specific activity; peaks 1 and 3 showed reduced activity and no activity was detected in the fourth peak. The elution pattern of the molecular weight markers is shown in figure 4.3; the second peak eluted between the molecular weight markers ranging between 30 to 100 K daltons.

The supernatant of ultracentrifuged AHC-AEF was found to be devoid of any AHC-AEF activity. The sediment however, after reconstitution with BBS to its original volume, demonstrated a comparable bioactivity as the crude AHC-AEF (table 4.1). Figure 4.4 shows the elution profile of the supernatant of AHC-AEF after ultracentrifugation. A comparison between figures 4.3 and 4.4.
Table 4.1

Effect of boiling, pH, ultracentrifugation and gel filtration on the bioactivity of AHC-AEF.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent surface area of spleen covered with amyloid (±S.D.)</th>
<th>No. of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.5</td>
<td>16.26 ± 1.3</td>
<td>6</td>
</tr>
<tr>
<td>pH 3.0</td>
<td>0.00 ± 0.00</td>
<td>6</td>
</tr>
<tr>
<td>pH 9.5</td>
<td>0.21 ± 0.14</td>
<td>6</td>
</tr>
<tr>
<td>boiling</td>
<td>0.00 ± 0.00</td>
<td>6</td>
</tr>
<tr>
<td>peak 1</td>
<td>4.21 ± 0.91</td>
<td>6</td>
</tr>
<tr>
<td>peak 2</td>
<td>15.9 ± 1.7</td>
<td>6</td>
</tr>
<tr>
<td>peak 3</td>
<td>1.1 ± 0.81</td>
<td>6</td>
</tr>
<tr>
<td>peak 4</td>
<td>0.00 ± 0.00</td>
<td>6</td>
</tr>
<tr>
<td>crude</td>
<td>16.9 ± 1.5</td>
<td>6</td>
</tr>
<tr>
<td>ultracentrifugation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) supernatant</td>
<td>0.00 ± 0.00</td>
<td>6</td>
</tr>
<tr>
<td>(b) sediment</td>
<td>13.5 ± 1.4</td>
<td>6</td>
</tr>
</tbody>
</table>
Figure 4.3

Ascending chromatography of AHC-AEF at 4°C on Sephacryl S-300 column equilibrated with borate-saline buffer, pH 7.4. Fraction volume was 2.5 ml, flow rate was 20 ml/hr, sample size was 3 ml (12 mg of protein/ml of AHC-AEF). The elution of the molecular weight markers are shown on the figure (from left to right: dextran blue, mouse IgG and myoglobin type 1 from equine skeletal muscle).

Figure 4.4

Ascending chromatography, at 4°C, of ultracentrifuged AHC-AEF on Sephacryl S-300 column equilibrated with borate-saline buffer, pH 7.4. The running conditions are similar to those in the above figure.
clearly shows the absence of peak 1 and a significant reduction in the heights of peaks 2 and 3.

**f. Effect of AHC-AEF on amyloid deposition in AHC-infected mice.**

The effect of concomitant injections of AHC-AEF and AHCs on the induction of amyloid deposition was tested in C57BL/6J, Balb/c and A/JAX mice. Mice in each group received 50 ± 10 AHCs i.p. and 1 ml of AHC-AEF. The amount of amyloid deposited in the splenic tissues of these mice after 1, 2, and 3 weeks p.i. are compared with mice infected with a similar dose of AHCs but did not receive AHC-AEF. (Table 4.2.) The results show that amyloid deposits appear in the splenic tissues of AHC-AEF treated and AHC-infected mice as early as one week after the infection. The amount of amyloid deposits in the splenic tissues were significantly higher at 2 and 3 weeks p.i. As has been shown in chapter 1 the group of mice which did not receive AHC-AEF did not become positive for amyloid until the fifth or sixth week p.i. The amount of amyloid in these mice at 12 weeks p.i., was comparable to the mice which had received AHC-AEF, and examined at 3 weeks post inoculation (Table 4.2).
Table 4.2

Effect of AHC-AEF on amyloid induction in AHC-infected C57BL/6J, Balb/c and A/JAX mice. Data represents the mean ± S.D. of cross-sectional areas of the spleen obliterated with amyloid deposits.

<table>
<thead>
<tr>
<th>mouse strain</th>
<th>treatment</th>
<th>% cross-sectional area of spleen covered with amyloid.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6J</td>
<td>A</td>
<td>26.3 ± 36.1 42.2 ± 4.35 N.D. N.D. N.D. N.D.</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>- - - - - 22.5 ± 38.7 ± 5.0 8.9 7.1</td>
</tr>
<tr>
<td>Balb/c</td>
<td>A</td>
<td>30.7 ± 5.2 37.8 ± 7.4 48.6 ± 4.8 N.D. N.D. N.D. N.D.</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>- - - - - 44.5 ± 55.5 ± 7.8 8.1 10.6</td>
</tr>
<tr>
<td>A/JAX</td>
<td>A</td>
<td>29.7 ± 4.1 34.3 ± 6.3 41.2 ± 4.8 N.D. N.D. N.D. N.D.</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>- - - - - 45.0 ± 9.1</td>
</tr>
</tbody>
</table>

WEIGHTS POST INFECTION

1 2 3 4 5 6 8 12

(A) + AEF
(B) No AEF
DISCUSSION.

At least two significant points emerged from the data presented in this chapter. First, the AHC-infected mice generate an AEF-like factor in their spleens and the bioactivity of this factor is comparable to that of azocasein- or silver nitrate-induced AEF. Second, the passive transfer of this factor to syngeneic or allogeneic mice followed by an inflammatory stimulus (silver nitrate or AHCs) hastened the process of amyloid deposition in the recipient mice. In the experiments presented in Chapter 1 it was shown that AHC-infection induces amyloidosis in C57BL/6J, CBA, Balb/c and A/JAX mouse strains. However, the time of appearance of amyloid deposits varied markedly between these strains. The data presented here show that AHC-AEF activity was absent from AHC-infected C57BL/6J mouse spleens at 4 weeks p.i. At this time period these spleens were negative for amyloid deposits. AHC-AEF activity was detected at 5 weeks p.i. in the spleen homogenates and the sediment from these spleens were positive for amyloid. The presence of perifollicular amyloid deposition was also confirmed in histological preparations. AHC-AEF activity was also detected in spleen homogenates from Balb/c and A/JAX mice at 5 and 12 to 16 weeks p.i. The observed resistance of the A/JAX mouse strain to amyloid induction has been attributed to a defective processing of SAA at the lysosomal level rather than decreased synthesis of the amyloid precursor (McAdam and Sipe, 1976; Wohlgethan and Cathcart 1979). However,
Axelrad et al. (1982) have shown that amyloid deposition is preceded by the appearance of AEF in host tissues. The data obtained in this study confirm their findings. It was observed that the time of appearance of AHC-AEF in hydatid-mice correlated with the time of appearance of amyloid fibrils. However, when AHC-AEF was passively transferred to AHC-infected C57BL/6J, Balb/c and A/JAX mice, the induction period of amyloid deposition was markedly reduced from 5 or 12 weeks to 1 week and the amount of amyloid in the splenic tissue was comparable to those infected for 12 weeks (Table 4.2).

When the AHC-AEF, obtained either from Balb/c or A/JAX mice, was transferred to C57BL/6J mice and followed by an inflammatory stimulus, of silver nitrate solution, the recipient mice became amyloidotic within 16-48 hours. The kinetics of amyloid deposition following the allogeneic transfer of AHC-AEF was identical to those observed after syngeneic transfer, indicating that AHC-AEF is not genetically restricted. Furthermore, the data show that the recipient of AHC-AEF does not require a challenge with the same inflammatory stimulus used to generate AHC-AEF in mice.

The properties of AHC-AEF were similar to those of AEF (Axelrad et al. 1982) with a few exceptions. Table 4.3 summarizes some of the salient physicochemical characteristics of AHC-AEF, AEF and the donor or transfer factors. Unlike AEF (Axelrad et al. 1982), the half life of AHC-AEF in vivo appears to be less than 24 hours and high doses of this factor result in a decline in the amount of amyloid deposited in the spleen. This
Table 4.3

A summary of the characteristics of the donor (transfer) factor and AEF from various sources and compared with AHC-AEF.

<table>
<thead>
<tr>
<th>Character</th>
<th>AHC-AEF</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>C57BL/6J</td>
<td>CBA</td>
<td>C57BL/10J guinea pigs &amp; peritoneal cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inoculum/mouse</td>
<td>10 mg protein</td>
<td>25 x 10^6</td>
<td>50-100 x 10^6</td>
<td>100 x 10^6</td>
<td>200 x 10^6</td>
</tr>
<tr>
<td>(no. of spleen)</td>
<td>25-50 x 10^6 sonicated spleen cells (100 mg of wet weight of spleen)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Route of administration</td>
<td>i.p.</td>
<td>i.v., i.p.</td>
<td>i.v.</td>
<td>i.p.</td>
<td>i.p.</td>
</tr>
<tr>
<td>Induction period</td>
<td>16-48 hr</td>
<td>24-48 hr</td>
<td>2 weeks</td>
<td>3-8 days</td>
<td>5 weeks</td>
</tr>
<tr>
<td>Inflammatory stimulus</td>
<td>AgNO₃, Azo-casein</td>
<td>AgNO₃, Azo-casein</td>
<td>nitrogen mustard</td>
<td>Azo-casein</td>
<td>cain</td>
</tr>
<tr>
<td>Subcellular localization</td>
<td>All subcellular fractions</td>
<td>T &amp; B-cell deprived spleen cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical nature</td>
<td>glycoprotein</td>
<td>glycoprotein</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Activity across present the allogenic histocompatibility barrier</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Effect of:

- Boiling: inactivated — — — —
- pH: inactivated at pH 3.5 inactivated at pH 3.5 — —
- Freezing: stable stable — —
- Freeze drying & dialysis: stable — —
- Fractionation: stable stable — —
- DNase: stable stable — —
- RNase: stable stable — —
- Trypsin: stable — — —
- Pepsin: stable — — —
- Protease: — inactivated — —

Note: Roman numerals and other symbols are used to denote specific effects or conditions.
Table 4.3 cont.

(A) Axelrad et al. (1982), (B) Harada et al. (1975), (C) Hultgren et al. (1967)
(D) Cathcart et al. (1972), (E) Janigan (1978), (F) Ranlov (1968)
(G) Shirahama et al. (1969), (H) Willerson (1969), (I) Hardt and Hellung Larsen (1972)

<table>
<thead>
<tr>
<th>Character</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source:</td>
<td>C3H/HeJ</td>
<td>C3H</td>
<td>Human with primary Swiss, C57BL/6J</td>
<td>C3H</td>
<td>C3H</td>
</tr>
<tr>
<td>Inoculum/</td>
<td>00-370 mg protein</td>
<td>100 mg of wet</td>
<td>500 mg of wet</td>
<td>300-500 mg of wet</td>
<td>100 \times 10^6</td>
</tr>
<tr>
<td>Mouse</td>
<td>weight of spleen</td>
<td>weight of spleen</td>
<td>weight of spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(No. of spleen</td>
<td>cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Route of</td>
<td>i.p.</td>
<td>i.v.</td>
<td>i.p.</td>
<td>i.v.</td>
<td>i.v.</td>
</tr>
<tr>
<td>Administration</td>
<td>4 days</td>
<td>4 days</td>
<td>5 days</td>
<td>4 days</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Induction</td>
<td>Azo-casein</td>
<td>nitrogen mustard</td>
<td>or casein</td>
<td>nitrogen mustard</td>
<td>nitrogen mustard</td>
</tr>
<tr>
<td>Period</td>
<td>or casein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td>Azo-casein</td>
<td>nitrogen mustard</td>
<td>or casein</td>
<td>nitrogen mustard</td>
<td>nitrogen mustard</td>
</tr>
<tr>
<td>Stimulus</td>
<td>or casein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcellular</td>
<td>Amonium</td>
<td>sulfate ppt.</td>
<td>nuclear fraction</td>
<td>nuclear fraction</td>
<td>nuclear fraction</td>
</tr>
<tr>
<td>Localization</td>
<td>or casein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical</td>
<td>globulin?</td>
<td>DNA, RNA</td>
<td>&amp; histones</td>
<td></td>
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</tr>
<tr>
<td>Nature</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>Activity across</td>
<td></td>
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<td></td>
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<tr>
<td>the allogenic</td>
<td>present</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>histocompatibility</td>
<td>present</td>
<td></td>
<td></td>
<td></td>
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<td>barrier</td>
<td>present</td>
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<tr>
<td>Effect of:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>a. boiling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. pH</td>
<td>inactivated</td>
<td>inactivated</td>
<td>inactivated</td>
<td>inactivated</td>
<td></td>
</tr>
<tr>
<td>c. freezing</td>
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<td>stable</td>
<td>stable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d. freeze</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>drying &amp;</td>
<td></td>
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</tr>
<tr>
<td>dialysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e. fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>f. DNAase</td>
<td>stable</td>
<td>stable?</td>
<td></td>
<td></td>
<td>inactivated</td>
</tr>
<tr>
<td>g. RNAase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>h. trypsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. pepsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>j. protease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>k. protease</td>
<td></td>
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</tbody>
</table>


discrepancy may be due to the difference in the methods of preparation between AHC-AEF and AEF as well as the route of administration in the test animals. Axelrad et al. (1982) administered spleen cell lysates intravenously in mice to test the bioactivity of AEF. Data presented in this chapter are based on the supernatant of spleen cell extract (30,000 x g) which are injected i.p. in mice. Therefore, it is logical to suggest that the water soluble AHC-AEF may be catabolized and cleared by the recipient mice more rapidly than the particulate spleen cell lysates. Furthermore, no regression or partial resolution of amyloid deposits occurred when the inflammatory stimulus was removed. In contrast, the amount of amyloid deposited in the splenic tissues increased significantly with time (Fig. 4.1). It was also shown that AHC-AEF is soluble in physiologic media and when chromatographed on Sephacryl-300 it eluted mainly in the second peak. The elution profile of the second peak was distributed between molecular weight markers of 30 to 100 K daltons and similar to AEF, the activity of AHC-AEF was pelleted at a centrifugal force equivalent to 250,000 x g for 2.5 hours. Since the AHC-AEF activity is sedimentable by ultracentrifugation and is found in the first three fractions on Sephacryl S-300, it is possible to suggest that active AHC-AEF molecules either aggregate or form heterocomplexes with other proteins in the solution. Axelrad et al. (1982) showed that AEF molecules bind, apparently irreversibly, to various intracytoplasmic components such as microsomes, ribosomes and to membrane fragments devoid of ribosomes and to inert particles such as iron filings.
As shown by Axelrad and Kisilevsky (1980), the treatment of AHC-AEF with RNAase, DNAase or lipase did not reduce its bioactivity significantly. The maximum reduction was observed following pronase or protease treatments. However, it is premature to make any definite conclusions from these preliminary observations about the chemical nature of AHC-AEF. The ineffectiveness of the enzymes may be attributed to the inaccessibility of the sites of action due to the tertiary structure of the protein.

The AHC-AEF is stable and soluble in physiologic media. No loss of activity was observed when the AHC-AEF was refrigerated at 4°C for 10 days or kept frozen for long periods of time (up to 2 years) either in tissue or as a tissue extract. These properties will allow the manipulation and purification of AHC-AEF without the loss of its bioactivity. Therefore, AHC-AEF appears to be an appropriate and important tool with which the pathogenesis of amyloidosis can be further investigated.

The data presented in this chapter does not clarify whether AHC-AEF is in fact AEF or the donor (transfer) factor. The information available on either of these amyloid enhancing factors is not complete and differences in the methodology of preparation and the amount of active material present in tissue extracts may account for the majority of the observed differences.
The data presented in this study extend previous observations and firmly establish the amyloidogenic potential of alveolar hydatid cysts to induce amyloidosis in various mouse strains (Ozeretskovskaya, personal communication; Ali-Khan et al. 1982; Mettler et al. 1982). In the four mouse strains examined (C57BL/6J, CBA, Balb/c and A/JAX) the AHCs grew relatively slowly for the first 6 to 8 weeks of infection and subsequently at an accelerated pace (table 1.1). Amyloid deposits appeared at 6 weeks p.i. in the susceptible mouse strains and 10-12 weeks p.i. in the resistant mouse strains. These results indicated that amyloidogenesis in the mouse strains examined did not correspond with the load of the parasite biomass. Amyloid resistance in the A/JAX strain is well established and is thought to be related to defective processing of serum amyloid A protein (SAA) at the lysosomal level rather than decreased synthesis of SAA (McAdam and Sipe, 1976; Wohlgethan and Cathcart, 1979). Based on the amount and duration of exposure time to casein, A/JAX mice have been shown to require a 3 to 4 fold longer exposure time to amyloid induction than CBA/J, C57BL/6J or Balb/c strains (Janigan and Druet, 1966; Cohen and Shirahama, 1972). However, the data presented in table (4.2) show that amyloid deposition in both the susceptible and the resistant mouse strains is solely dependant on the generation of an amyloid enhancing-like factor (AHC-AEF), which was isolated from spleens of AHC-infected mice at 5, 8 or 12 weeks p.i. The transfer of AHC-AEF to any of the 4 mouse
strains examined resulted in amyloid deposition following the administration of AHCs as the inflammatory stimulus, and the amount of amyloid in the splenic tissues of these mice after 1 week was comparable to those infected for 12 weeks with AHCs only (table 4.2). It is important to emphasise on two significant points that emerged from the data presented on AHC-AEF. First, the AHC-infected mice generate an AEF-like substance in their spleens and the bioactivity of this factor is comparable to that of azocasein- or silver nitrate- induced AEF. Second, the passive transfer of this factor to syngeneic or allogeneic mice followed by an inflammatory stimulus (silver nitrate or AHCs) hastens the process of amyloid deposition in the recipient mice, including the amyloid resistant mouse strain.

There is no information available in the literature about the AHC-elaborated pathogenetic factors. The work presented in this study identified for the first time at least four important biological properties of AHC-antigens. They induce inflammation and promote neutrophil accumulation at the site of injection, possess chemotactic activity for neutrophils and macrophages, Kill normal splenocytes in vitro and induce amyloid deposition in the spleens of mice. These activities were found to be dose and time dependant. The phlogistic and chemotactic activities of AHC-antigen is not surprising. The injection of AHCs, either intraperitoneally or subcutaneously into mice induces a prompt and intense inflammatory response at the site of cyst inoculation. The influx of cells is progressive and persists throughout the course of infection (Ali-Khan and Siboo, 1980;
Devouge and Ali-Khan, 1983; Treves and Ali-Khan, 1984a). However, the cytotoxic and amyloidogenic properties of the AHC-antigen are very interesting. Although the precise mechanisms that lead to amyloid deposition following the AHC-antigen administration is not known, the current study firmly establishes the role of the parasite in the induction of secondary amyloidosis.

The AHC-antigen used in the present study is a soluble extract of purified AHCs that contains soluble somatic parasite antigens (hidden somatic antigens), metabolic proteins and enzymes. These antigens would not normally be present in large concentrations in the host environment except focally when cysts are destroyed by the effector cells of the immune system during the initial stage of infection. This process would result in the release of amyloidogenic somatic parasite antigens in the host environment at very low concentrations. The continuous release and persistence of these antigens, as evidenced by the generation of circulating immune complexes in the host (Ali-Khan and Siboo, 1980; Treves and Ali-Khan, 1984b), over a period of 5 to 6 weeks might result in the generation of AHC-AEF and eventually amyloid deposition. Based on these premises it is therefore logical to assume that the introduction of somatic AHC-antigens at high concentrations in mice results into overt amyloid deposition within 48 hours. On the other hand, it is also possible to assume that the AHC-antigen preparation contained AHC-AEF which was responsible for the rapid amyloid deposition. However, the following reasons support the first proposal. First, AHC antigen, unlike AEF, does not require concomitant administration of a
potent inflammatory stimulus, such as silver nitrate, for amyloid induction (Axelrad et. al. 1980). Second, the AHC-antigen, as mentioned earlier, is an extract of cysts that does not contain host tissues such as spleens or livers which normally would contain AHC-AEF. Third, the amount of protein injected is far below the minimum amount required for AHC-AEF to exert any effect in the host with a potent inflammatory stimulus (see fig. 4.1).

The AHC-induced amyloid protein has been partially characterized. It is a small Mw protein (8000-9900 daltons) whose pI value ranges between 5.1-5.3. On SDS-PAGE, it appears as a doublet and is identical to that of casein induced protein AA. The immunologic cross-reactivity between AHCA and AZCA was determined by immunodiffusion, immunofluorescence and immunoperoxidase tests. Furthermore, following immunoblotting on nitrocellulose paper, either antiserum reacted with the AHCA protein. These findings clearly suggest that AHCA is antigenically similar to AZCA and thus is indeed an AA-type of amyloid. However, AHCA differed from AZCA in pI values, resistance to KMnO₄ and trypsin treatments and amino acid composition. AHCA was shown to have an amino acid composition closer to that of age associated or senescence accelerated type of amyloid (Scheinberg et. al. 1976a; Matsumura et. al. 1982). Despite differences in the amino acid composition between different mouse amyloid proteins (see table 2.3) aspartic, glutamic, glycine and alanine were present in high proportions in all of the amyloid proteins including the AHCA. These differences might account for
the variation in pI value and resistance to KMno treatment.

Finally, casein-induced amyloidosis in mice is pathologically and pathophysiologically similar to human secondary amyloidosis. The primary interest of the alveolar hydatid cyst mouse model in this context is that it provides an appropriate alternative to study the relationship between circulating immune complexes, recurrent inflammation, hyper gammaglobulinemia (Ali-Khan and Siboo, 1980, 1982, 1983), depressed delayed hypersensitivity response (Ali-Khan, 1978b; Devouge and Ali-Khan, 1983) and secondary amyloidosis. All the above at one time or another, have been incriminated as the pathogenetic factors in the induction of amyloidosis. In addition, it can now be stated with certainty that the present model is predictable and the induction of amyloidosis is virtually 100% in both the susceptible and resistant mouse strains following an experimental infection or the injection of parasite antigens.
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