Evidence of oxidative stress response in a mouse model of AA amyloidosis: Immunolocalization of specific markers

by

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ABSTRACT

M.Sc. Thesis  Golnar Kamalvand  Microbiology and Immunology

Amyloidosis describes a heterogeneous collection of systemic diseases characterized by the extracellular deposition of proteinaceous amyloid fibrils derived from normally soluble proteins. With progressive tissue deposition of amyloid, death can occur through failure of the affected organ(s). However, the mechanism of amyloid fibril formation remains obscure. To understand this mechanism, a parasite (alveolar hydatid cyst, AHC)-mouse model of inflammation-associated AA amyloidosis, was used. AHC is a potent inducer of chronic inflammation, serum amyloid A (SAA) synthesis and AA amyloidosis. It has been proposed that reactive oxygen radicals (ROR) generated by inflammatory macrophages (mΦ) and reticuloendothelial (RE) cells, which are also intimately involved in SAA clearance, could initiate intra-lysosomal AA fibril formation. ROS generated intracellularly could oxidize SAA fragments or other key cellular proteins, alter them structurally and thus render them resistant to enzymatic degradation and prone to intralysosomal nascent AA fibril formation.

The objective of this research was to identify oxidative stress (OS) markers (heme-oxygenase-1, HO-1; 4-hydroxy-2-nonenal, HNE; N\(^{\circ}\)-(Carboxymethyl)lysine, CML) in peritoneal mΦ and splenic/hepatic RE cells obtained from AHC-infected mice prior to and during AA amyloidosis. Histochemical and peroxidase-immunoperoxidase methods were used to detect the OS markers. High levels of HO-1, an antioxidant enzyme; HNE, a product of lipid peroxidation; and CML, an advanced glycation end product, were found in peritoneal mΦ and splenic/hepatic RE cells proximal to AA fibril deposition. HNE and CML deposits were found in both the tissue interstitium and bound
to AA amyloid deposits, indicating their possible role in the oxidative alteration of intracellular SAA. OS mediated changes in mΦ/RE cells loaded with SAA may prove to be a prelude to nascent intracellular AA fibril formation.
L’amylose consiste en la voie finale d’une collection hétérogène de pathologies systémiques caractérisées par un dépôt amyloïde. Le dépôt protéique extracellulaire fibrillaire anormal provient d’une source normalement soluble. En progressant, le dépôt amyloïde entraîne le mauvais fonctionnement des organes affectés ce qui peut provoquer la mort. Si ce phénomène est déjà amplement traité dans la littérature, le mécanisme même de la formation de fibrilles amyloïdes demeure obscur. Afin de mieux saisir les piliers de ce mécanisme, cette étude utilise un modèle de souris de l’amyloidose AA infecté de kystes hydatiques alvéolaires (KHA). Le KHA est un puissant catalyseur d’inflammation chronique et de synthèse du sérum amyloïde A (SAA), lequel le est précurseur sérique de l’amylose AA.

Les radicaux libres oxygénés (RLO), générés suite à l’activation inflammatoire des macrophages (mΦ) et des cellules réticulo-endothéliales (RE) jouent un rôle d’importance dans la dégradation de SAA. Aussi cette recherche s’articule-t-elle autour du prémisse que ces radicaux puissent initier la formation de fibrilles intralysosomale AA. Les RLO, générés de façon intracellulaire pourraient oxyder les fragments de SAA, de même que d’autres protéines cellulaires clés. Altérant la structure protéinique, la présence de RLO se traduirait par une résistance des protéines à la dégradation enzymatique, d’où la formation initiale de fibrilles intralysosomales AA.

L’objectif de la présente recherche fut d’identifier des marqueurs de stresse oxydant (SO) (heme-oxygénase-1, HO-1; 4-hydroxy-2-nonénal, HNE; Nε - (Carboximéthyl)lysine, CML) dans les mΦ inflammatoires péritoneaux et les cellules RE
spléniques/hépatiques des souris infectées de KHA avant et pendant le dépôt des fibrilles AA. Les méthodes histochimiques et la méthode en immunopéroxydase furent utilisées afin que soient détectés les marqueurs de SO. Conformément à l’hypothèse initialement formulée, de hauts niveaux d’enzyme anti-oxydant HO-1, du produit de péroxydation lipidique HNE et d’un produit final de glycolysation avancée CML furent décelés dans les mΦ péritoneaux et les cellules RE spléniques/hépatiques à proximité des fibrilles AA.

La protéolysation de dépôt HNE et de CML s’effectua tant dans les tissus interstitiaux que dans ceux liés aux dépôts amyloïdes AA, d’où la proposition de l’hypothèse d’un rôle actifs de ces marqueurs de SO dans l’altération oxydative du SAA intracellulaire. Des changements liés aux SO dans les cellules mΦ/RE chargées de SAA pourraient s’avérer être la preuve du rôle clé du SO dans le prélude de la formation des fibrilles intracellulaires AA.
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CONTRIBUTION OF AUTHORS

This master’s of science thesis contains two original papers, one that is published and one that has recently been submitted, as Chapters 2 and 3 of this thesis. Each of these chapters contains their own Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements and References sections. Prefaces that serve as connecting texts to bridge the published papers are found prior to Chapters 2 and 3. A general Introduction (Chapter 1) and a section that contains Conclusions and Discussion (Chapter 4) are also included. References in Chapters 1 and 4 are collated alphabetically whereas in published papers they are listed in the order they appear within the text of the manuscripts. Page numbers of the thesis are found on the bottom center of each page and should be distinguished from the page numbers of individual manuscripts.

The published papers that comprise the chapters describing the experimental data of this thesis are listed below. I am the principal author in both papers. My research was conducted under the supervision of Dr. Z. Ali-Khan, who helped in composing the text of both papers. Some experiments in Chapter 2 were performed in collaboration with Geneviève Pinard who works as a technician in our laboratory.


CHAPTER 1. LITERATURE REVIEW, RATIONALE AND OBJECTIVES OF THE THESIS

1. AMYLOIDOSIS – A PROTEIN FOLDING DISORDER

Amyloidosis is a disorder of protein metabolism. The term ‘amyloid’ is a generic term used to describe extracellular, fibrillar protein deposits associated with disease. The fibrils are insoluble and generally resist proteolytic digestion. They aggregate to indefinite lengths in organs and tissue, and through pressure atrophy, destroy the surrounding normal tissues. Some of these disorders are systemic while in other forms the amyloid deposition is restricted to particular organ systems (Husby, 1992). Clinical manifestations of amyloidosis vary widely and depend on the organs involved (Stone). Amyloid deposits exert their pathological effects largely by their physical presence, distorting tissue architecture and disrupting organ function. Amyloidosis accompanies and is associated with a number of medical disorders, including adult-onset diabetes, rheumatoid arthritis (RA), chronic renal dialysis, familial amyloid neuropathy and most notably Alzheimer’s disease (AD), to name a few. Therapy is remarkably deficient and can mean a fatal outcome, primarily due to organ failure (Tan; Kisilevsky, 1983).

A necessary condition for the formation and deposition of amyloid fibrils is the presence of an autologous protein precursor that is abnormal in structure and/or concentration. The modern classification of amyloidosis is based on the nature of the precursor plasma protein (Falk). More than 20 forms of amyloid are now recognized (Westermark); the incidence of these diseases range from being rare to playing central roles in the pathogenesis of diseases affecting millions of patients (i.e. AD, adult-onset diabetes). The different amyloid types can be distinguished immunohistochemically with
specific antibody against the precursor and/or amyloid protein itself. Table 1 lists a few of the precursors and the diseases they cause (Bellotti); among the precursors are plasma constituents (serum amyloid A, Ig light chain, fibrinogen, transthyretin, and apolipoproteins) or precursors that form localized amyloids (β-protein precursor, calcitonin, cystatin C, and atrial natriuretic factor).

Although amyloid fibrils are derived from a variety of precursor proteins in different forms of the disease, they share characteristic tinctorial properties. Amyloid fibrils have specific affinity for thioflavin and, when stained with Congo red, they display an apple-green birefringence under polarized light. Amyloidogenic proteins for different amyloids are distinct with respect to amino acid sequence, however, all fibrils are structurally similar. For example, electron microscopy and X-ray diffraction patterns reveal that amyloid fibrils have a diameter from 5-13 nm and form rigid, unbranched, cross β-pleated sheets (Glenner). Interestingly, this β-pleated sheet structure is not normally found in this pure conformation in mammalian tissues, but is found in invertebrates (Glenner). Their common structural properties imply that amyloid fibrils have a common mechanism of fibrillization. Consequently, much effort has been directed towards understanding the fibrillogenesis pathway, with the aim of developing inhibitors to treat various amyloidoses.
Table 1. Nomenclature and classification of amyloidosis

<table>
<thead>
<tr>
<th>Amyloid Protein</th>
<th>Protein Precursor</th>
<th>Clinical syndrome</th>
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| AA              | serum amyloid A (SAA) | - Reactive (secondary) amyloidosis associated with recurrent inflammation (i.e. rheumatoid arthritis)
|                 |                  | - Familial Mediterranean fever
|                 |                  | - Familial amyloid neuropathy |
| AL              | Ig light chain | - Idiopathic primary amyloidosis |
| AH              | Ig heavy chain | - Idiopathic primary amyloidosis |
| ATTR            | transthyretin | - Familial amyloid neuropathy
|                 |                  | - Familial amyloid cardiomyopathy
|                 |                  | - Senile systemic amyloidosis |
| AApoI           | apo A1 variants | - Atherosclerosis |
|                 |                  | - Familial neuropathic and non-neuropathic amyloidosis |
| ACys            | cystatin C variants | - Hereditary cerebral hemorrhage |
| Aβ2M            | β2-microglobulin | - Associated with chronic renal dialysis |
| Aβ              | β-precursor protein | - Alzheimer’s disease |
|                 |                  | - Down’s syndrome |
|                 |                  | - Hereditary cerebral hemorrhage |
| AANF            | atrial natriuretic factor | - Isolated atrial amyloid |
| ACaI            | (pro)calcitonin | - Medullary carcinomas of the thyroid |
2. CLINICAL ASPECTS OF AA AMYLOIDOSIS

Reactive or AA amyloidosis is brought upon by chronic inflammatory disorders that provoke a sustained acute phase response. The first 24-48 hours of inflammation are known as the acute-phase response, characterized by a dramatic increase in hepatic synthesis of acute phase proteins (Gabay). During chronic inflammation, acute phase protein levels, after an initial increase, decrease to steady-state concentrations that are significantly higher than the original baseline levels. Thus, chronic inflammation can result in continuing tissue damage and, occasionally, complications such as secondary AA amyloidosis. This form of amyloidosis is characterized by the tissue deposition of AA fibrils derived from serum amyloid A (SAA) (Glenner).

Many chronic inflammatory disorders of known and unknown etiologies are known to predispose to AA amyloidosis. Chronic inflammatory conditions may arise from prolonged microbial or parasitic infections (i.e. leprosy, tuberculosis, leishmaniasis, malaria, and alveolar hydatid disease), malignant neoplasm (i.e. Hodgkin’s disease and renal carcinoma), and chronic inflammatory disorders including RA, juvenile chronic arthritis, psoriasis and Crohn’s disease (Benson, 1995; Glenner). In developed countries, where chronic infections such as tuberculosis and leprosy are for the most part contained, RA is the most frequent predisposing disease, with an estimated 1-10% of incidence of amyloidosis in RA patients (Glenner, Tan).

While the presence of SAA at high levels is known to be a major predisposing factor to AA amyloidosis, it is not sufficient on its own to cause amyloid deposits. Only a limited number of RA patients ranging from 1 to 10% develop AA amyloidoses. Etiology and pathogenesis of AA amyloidosis, and other forms of amyloidosis, are multifactorial.
Genetic susceptibility could be a significant factor; while the frequency of amyloidosis in RA patients in Caucasians is 1-2%, approximately 10-15% of Japanese RA patients develop the disease. Yamada et al. have recently shown that certain genetic backgrounds, particularly SAA polymorphisms, are associated with development of AA amyloidosis (Yamada, 2003). Their data suggest that a single nucleotide polymorphism within the SAA 5’ flanking region of the gene is related to amyloid development; their data correlated with differences seen in the frequency of AA amyloidosis in Japanese and Caucasian ethnic groups (Yamada, 2003). The means by which this allele may regulate susceptibility has yet to be determined.

Furthermore, there is considerable variability between individual cases with respect to distribution of amyloid deposits and clinical symptoms. Although the liver and spleen are the first sites of AA fibril deposition, the kidney is the most common site leading to clinical signs of disease (Stone). Patients with splenic/hepatic amyloid deposits can remain asymptomatic. Amyloid deposition in the kidney usually begins in the glomerular mesangium and around capillary basement membranes, leading to progressive obliteration of capillary lumina, destruction of glomerular cells and, eventually, complete replacement of glomerulus by a confluent mass of amyloid. Amyloid deposition in the kidney is characterized by proteinuria that can result in chronic renal failure. The gastrointestinal tract can also be involved and life-threatening gastrointestinal bleeding can occur. Amyloid infiltration in blood vessel walls can cause increased risk of hemorrhage and involvement of other organs, leading to death (Benson, 1995).

Unfortunately, the diagnosis of amyloidosis is usually made when patients already have advanced disease with resultant compromise in organ function. Hemodialysis or
peritoneal dialysis may prolong life. Suppression of the underlying disease process by alkylating agents in rheumatoid arthritis and juvenile chronic arthritis has been shown to preserve renal function and improve survival in patients. Chronic colchicine administration has been shown to prevent amyloidosis in patients with familial Mediterranean fever (Tan). In microbial or parasitic infections, treating the primary inflammatory disease with antibiotics is the key. The length of survival with AA amyloidosis depends on how early in the course of disease the diagnosis is made.
3. A PARASITE MODEL OF AA AMYLOIDOSIS

The discovery that alveolar hydatid cyst (AHC)-infected mice develop AA amyloidosis was made in the early 1980s by Ali-Khan et al. (Ali-Khan, 1982). Upon microscopic observation, susceptible mice infected intraperitoneally with 50-250 AHC, the larval stage of *Echinococcus multilocularis*, between 1 to 12 weeks post-infection (p.i.) depending on the infective inoculum, demonstrated hyaline eosinophilic areas in the spleen identified as AA amyloid deposits (Alkarmi, 1986). Despite a prompt influx of inflammatory cells at the inoculation site and heavy leukocyte infiltration into the tissue matrix, AHC grows like a tumor in various soft organs and potentially metastasizes (Treves). The infected mice do not appear to contain the infection (Ali-Khan, 1996). The proliferating AHC acts as a potent amyloidogen, with the sustained overproduction of SAA resulting in AA fibril formation.

*Echinococcus* is a small endoparasitic flatworm belonging to Class Cestoda. It exhibits an indirect life cycle in which the adult is hermaphroditic and the larva proliferates asexually, requiring two mammalian hosts for completion of its life cycle: a definitive host in which the adult worm develops in the small intestine, and an intermediate host in which the cysts develop in the viscera. The parasite is of pathogenic and economic significance in intermediate hosts, where the larval parasite develops into a hydatid cyst. Infection with *Echinococcus* may be naturally transmitted from the definitive host to man; ingestion of eggs in contaminated food or drink is the precursor of alveolar hydatid disease (Rausch).

*Echinococcus multilocularis* exhibits a mainly holarctic distribution with foxes primarily as the definitive host, and rodents and man as intermediate hosts; central North
America has however become a niche for *E. multilocularis* since the 1960s. The unique feature of this species is the multivesicular (alveolar) nature of its metacestode; it is an infiltrating structure, with no limiting host-tissue barrier, consisting of numerous small vesicles embedded in a dense stroma of connective tissue, localized initially in the liver (Rausch). Furthermore, the detachment of germinal cells and their subsequent distribution via the lymph and blood can give rise to the distant metastatic foci characteristic of *E. multilocularis*. Clinical and pathological changes in experimentally infected rodents include hepato/splenomegaly, increase in body weight due to the proliferating metacestode, ascites and finally death within five months of infection (Torgerson). In essential features, the course of AHC-infection in rodents appears to be a wasting disease. Upon post-mortem examination, infiltration of the liver, peritoneal cavity, other abdominal organs and the lungs may be evident (Fig. 1).

**FIGURE 1.** Abdominal view of a post-mortem alveolar hydatid cyst (AHC)-infected mouse
The reactivity of the spleen in response to the AHC-infection appears to play an important role in the clinical features and the course of the disease. Briefly, the spleen is a bi-functional, complex organ that acts as a hematopoietic vascular filter and functions in the reticular defense system. The splenic parenchyma consists of three parts: the white pulp, is rich in lymphocytes and the site of germinal centers; the red pulp, typically the largest part of the spleen, which stores and destroys erythrocytes, is site of a reticular meshwork; and the perifollicular zone, lying between the white pulp and red pulp, which contains large venous sinuses, is the first site for antigen and lymphocyte deposition (Weiss, 1978a). The reticuloendothelial system is an extensive system of protection and regulation whose prominent feature is phagocytosis. Macrophages (MΦ) are large-capacity phagocytes, as well as secretory cells. MΦ synthesize and secrete collagenase, components of complement, and inflammatory cytokines. Lysosomes (LY), upon secretion, provide a bath of acid hydrolytic enzymes within MΦ that digest phagocytosed substances. This is notable in the red pulp (Weiss, 1978b).

Elevated SAA level, expansion of the reticuloendothelial cells (monocytoid cells in the splenic perifollicular zones and Kupffer cells in the liver) and localization of SAA in the endosomes-lysosomes of these cells are seen in the pre-amyloidotic phase (Chronopoulos). The primary targets of amyloid deposition are thus the perifollicular zone sinuses in the spleen, and portal and central veins and sinus walls in the liver (Alkarmi, 1984). Progressive amyloid deposition between 1 week and 12 weeks p.i. significantly distorts the splenic architecture as well as causes atrophic changes in the liver, kidneys and GI tract (Ali-Khan, 1983 and 1996).
The AHC-infected mouse is a useful in vivo model to investigate the pathogenesis of AA amyloidosis. The azocasein mouse-model, necessitating daily subcutaneous injections of the chemical amyloidogen azocasein and producing amyloidosis in susceptible mice from 9 days to 3 weeks, is the most commonly used model to study the pathogenesis of AA amyloidosis (Janigan; Sipe, 1993). AHC, however, is a more potent amyloidogen; with a single infective inoculum of 250 AHC, the amyloid induction period is relatively short – about 1 week p.i. Both the preamyloidotic and the amyloidotic phases can be studied within a reasonably short time.

In addition, the availability of mouse strains that differ in susceptibility to AA amyloidosis provides a valuable tool to study factors that are potentially important in AA amyloidogenesis (Ali-Khan, 1988; Alkarmi, 1984). In susceptible mice, two acute-phase SAA proteins, SAA 1.1 (previously SAA2) and SAA2.1 (previously SAA1) are synthesized; only the SAA1.1 is amyloidogenic (Sipe, 1999). The CE/J mouse strain is absolutely resistant to AA amyloidosis (Sipe, 1993). The CE/J mouse contains a single SAA gene, coding for SAA2.2 (previously SAA_{CE/J}), which seems to be a composite of SAA1.1 and SAA2.1 genes (Yu). While SAA2.2 differs by only 6 amino acids from amyloidogenic SAA1.1, the differences lie in the amino terminal region of SAA1.1 responsible for amyloidogenicity, thus rendering the CE/J mouse amyloid resistant (de Beer). Thus, the CE/J AHC-infected mouse can serve as a valuable tool in studying the consequences of SAA deposition independent of AA fibril formation.
4. PATHOGENESIS OF AA AMYLOIDOSIS

In AA amyloidosis, high expression and aberrant catabolism of SAA are the main disposing factors to AA pathogenesis. In response to tissue trauma or an inflammatory stimulus, activated monocytoid cell-derived cytokines, primarily interleukin (IL)-1, IL-6 and tumor necrosis factor-α (TNF-α), induce the synthesis of acute phase proteins by liver hepatocytes at the transcriptional level (Benson, 1980; Zahedi). SAA may reach plasma levels up to 1000-fold greater (1 mg/ml) than that found in the non-inflammatory state. Serum SAA is mainly complexed to high-density lipoproteins (HDL) by displacing apolipoproteins (apo), apo A-I and A-II, and circulates as acute phase HDL-SAA complex (Husby, 1994).

SAA, in fact, represents several isoforms encoded by a family of highly conserved genes found across several mammalian species. While six murine SAA isoforms have been described, SAA1.1 and SAA2.1 are the prominent acute phase isoforms, produced in equimolar concentrations during the early phases of inflammation (other SAA isoforms are constitutive and only ever expressed in minor amounts). While these two isoforms share 95% structural identity, differing by only six amino acids, SAA1.1 is more rapidly cleared from the serum and much more prone to amyloidogenesis (Kluve-Beckerman, 1997; Bell, 1996). Biochemical analysis of purified SAA and AA proteins has confirmed that the N-terminal two-thirds of SAA1.1 form AA amyloid (Husby, 1994). The pathophysiology of this process, however, is not understood.

Aberrant proteolytic processing of amyloid precursor proteins by activated mΦ, RE cells or microglia has been implicated as a causative factor in many forms of amyloidosis, with most amyloid fibrils being truncated forms of larger precursors
(Kisilevsky, 1994). Whereas normal unstimulated monocytoid cells successfully degrade SAA within 4 hours, chronically activated monocytoid cells (i.e. AHC-derived) degrade SAA differentially, generating N-terminally intact AA-sized SAA derivatives (Gollaher; Bell, 1999). Cathepsin B, a major lysosomal cysteine proteinase, has been linked to the partial degradation of recombinant SAA and to the generation of the most common form of N-terminally intact AA-sized peptide of ~8 kDa (Yamada, 1995). The plausible link between these intracellular SAA fragments and extracellular AA fibrils was recently established by Kluver-Beckerman et al. (Kluve-Beckerman, 1999). They demonstrated that both native and recombinant murine SAA, co-cultured with mΦ, were concentrated in LY, generated intracellular N-terminal SAA fragments, and formed both intra- and extracellular AA fibrils (Kluve-Beckerman, 1999). In sum, SAA, endocytosed by a yet unidentified receptor on the surface of mΦ/RE cells, is sequestered into the LY in these cells, where its partial degradation yields amyloidogenic AA-sized fragments.

In AA amyloidosis, the splenic PFZ, populated by a diverse monocytoid cell population, is invariably the first site of AA deposition, followed shortly after by the liver, then the kidneys (Du). Fundamental pathogenetic aspects of amyloidosis appear to rely on local tissue conditions, components or events interacting to create the necessary conditions for amyloid formation. The extracellular expansion of amyloid fibrils is generally thought to occur via a nucleation-dependent oligomerization, accelerated by a ‘seeding’ mechanism (Rochet; Ali-Khan, 2002). In vitro studies show that the addition of a preformed nucleus or ‘seed’ to a supersaturated protein solution accelerates fibrillization compared with spontaneous self-assembly of fibrils. This phenomenon was confirmed in vivo in mice undergoing AA amyloidosis; AA fibril deposition was
markedly accelerated when the animals were given, in addition to an inflammatory stimulus, an intravenous injection of amyloid enhancing factor (AEF), a concoction of proteins extracted from AA fibril-laden tissue (Abankwa, Axelrad). Recently, the active principle of AEF was shown to be the amyloid fibril itself, effective at minute doses of <1 ng, showing transmittability akin to prion-associated disorders (Lundmark). Further experiments on murine AA amyloidosis demonstrated cross-nucleation, where fibrils derived from one form of amyloid was able to serve as a nucleus for another (Ganowiak, Johan).

Recently, in an effort to elucidate functions of SAA, a compelling finding relating SAA to extracellular AA fibrils was made. Aggregates of SAA, assuming a β-sheet conformation, were found to form channels in planar bilayer membranes, and thus could potentially form such channels in the LY and plasma membranes (Hirakura). As these channels are relatively non-selective, remain open for long periods and are quite large, they may represent the outlet for nascent AA fibrils into the interstitium, where nucleation-dependent aggregation can ensue. The precise molecular determinants necessary for the initial fibrillization remain elusive (Lansbury Jr.). Environmental factors are thought to be key, including ionic strength, pH and oxidation, in influencing protein folding and nucleation (Rochet). Clearly, the elucidation of the fibrillization pathway is essential to guide ongoing research in amyloidosis research.
5. PUTATIVE ROLE OF INFLAMMATION-INDUCED OXIDATIVE STRESS IN AMYLOID FORMATION

The production of intracellular reactive oxygen radicals (ROR) occurs as a ubiquitous byproduct of both oxidative phosphorylation and the myriad of oxidases necessary to support aerobic metabolism. The intracellular level of oxidized protein reflects the balance between the rate of protein oxidation and the rate of oxidized protein degradation. This balance is a complex function of numerous factors that lead to the generation of ROR, on the one hand, and of multiple factors that determine the activities of the proteases that degrade oxidatively modified proteins (Berlett).

In chronic inflammation, the increased production of cytokines (i.e., IL-1, IL-6, IL-18, M-CSF, and TNF-α) recruits and activates monocytes that in turn produce high concentrations of ROR (Gabay, Maury, Migita). In AA amyloidosis, the situation seems to be exaggerated by SAA and AA fibrils. Recently, Yan et al. showed that SAA1.1 and AA fibrils bound to murine BV-2 cells through the receptor for advanced glycation end products (RAGE) and induced expression of several pro-inflammatory cytokines (Yan). Furthermore, acute phase SAA1.1 was shown to enhance the biosynthesis of cyclooxygenase metabolites, potent mediators of inflammation, in activated monocytes up to 3-fold (Malle). Thus, inflammation and oxidative stress (OS) act in concert and perpetuate one another.

The principal damaging intermediates of OS response are the hydroxyl radical, the superoxide anion and hydrogen peroxide, highly reactive species that alter macromolecules present at their site of generation (Markesbery). ROR have been implicated in the modification of lipids, proteins and DNA, lysosome membrane damage
and fragmentation/unfolding of cellular proteins. Protein modification, a recognized manifestation of OS, has been extensively studied in the etiology of AD.

Evidence that ROR production is involved in the pathogenesis of AD is ever increasing (Markesbery). Oxidative modification of amyloid β protein (Aβ), which is a 39- to 42-amino acid peptide cleaved from a longer amyloid precursor protein (APP), may be an early event in Aβ pathogenesis and may be important in amyloid plaque formation. As described for AA amyloidosis, microglial cells (monocytes of the brain) internalize Aβ within lysosomal compartments in their effort to degrade the molecule (Wegiel). Consequently, while sequestered intracellularly, Aβ may be exposed to the activity of enzymes that together with ROR oxidatively modify Aβ. To this end, Head et al. performed immunohistochemical studies using antibody against oxidized Aβ1-40 in order to characterize the distribution of oxidized Aβ in AD brains. Their results implied a role for microglial cells in forming oxidatively cross-linked Aβ, which is highly aggregated and less amenable to degradation and clearance (Grune). They hypothesized that oxidatively modified Aβ may serve as a seed for the further deposition of unmodified, soluble Aβ into plaques (Head).

The fact that oxidative stress plays an important role in AD pathogenesis seems clear, given all the evidence that research has recently provided. The cytopathologic significance of oxidative damage is seen by the upregulation of antioxidant enzymes. Heme-oxygenase-1 (HO-1) is among the most sensitive indicator of cellular oxidative stress response and in AD, HO-1 response co-localized with amyloid deposits in the brain (Pappolla; Takeda, 2000a). The net effect of ROR is damaging; upregulations of OS markers found in AD brains include advanced glycation end-products (AGE) (Sasaki),
nitration (Smith, 1994a), lipid peroxidation adducts (Sayre, 1997a), as well as free carbonyls (Smith, 1996). As in AA amyloidosis, Aβ exaggerates the OS response; it has been shown that neuronal and microglial OS can be induced by Aβ by interaction with the receptor for advanced glycation end products (RAGE) (Sayre, 1997b). It has not been firmly established whether OS is directly involved in amyloid formation \textit{in vivo}, be it AA or Aβ amyloid, or if the amyloid fibrils, once formed, trigger an OS reaction. By contrast, \textit{in vitro} studies have generated both neurofibrillary tangles (NFT), a hallmark of AD, and Aβ from the precursor exposed to oxidation (Dyrks, Schweers). The protein modulating properties of ROR, present in amyloid deposits, increase the likelihood that they are involved in fibril formation.
6. RATIONALE AND OBJECTIVES

Activated mΦ/RE cell-mediated degradation of SAA is the primary clearance mechanism of exogenous SAA in vivo. An incessant flow of SAA into these cells, however, cannot be sustained without causing disturbances in the LY-mediated clearance of SAA. The resulting retention of partially degraded, N-terminally intact, SAA fragments in the LY can lead to the modification of such fragments. Acid conditions, in synergy with ROR generated in activated mΦ/RE cells, could precipitate intra-LY nascent AA fibril formation (Ali-Khan, 2002). Such fibrils, after their release, either by exocytosis or toxic cell death, could act as a nidus in the nucleation-dependent aggregation of extracellular AA fibril deposits. OS is an established underlying process during prolonged inflammation. The potential role of ROR in AA fibril formation remains obscure; oxidative changes in mΦ-derived SAA and in amyloidotic tissues need to be determined.

Using the AHC-infected mouse, a fully characterized model of inflammation-associated AA amyloidosis (Ali-Khan, 1996), we show for the first time OS response prior to and during AA amyloidosis. MΦ and splenic/hepatic tissues from pre-amyloidotic and amyloidotic phases were used to immunohistochemically localize markers of oxidative stress. The expression/generation of three such markers were monitored in AHC-derived tissues prior to and during AA fibril deposition: heme-oxygenase-1 (HO-1), an antioxidant enzyme; 4-hydroxy-2-nonenal (HNE), a product of lipid peroxidation; and Nε-(carboxymethyl)lysine (CML), an advanced glycation end product. The following two chapters present our encouraging results and validate our working hypothesis.
PREFACE TO CHAPTER 2

Virtually all organisms respond to environmental stress by redirecting their protein synthetic machinery to produce a small set of proteins termed heat shock proteins (HSP). HO-1, also known as HSP32, is induced in a variety of stressed states including exposure to heavy metals, UV light, hyperthermia, hypoxia, inflammation and OS (Maines). HO-1 catalyzes the first and rate-limiting step in the degradation of heme, a ubiquitous iron-containing compound essential for the activity of all aerobic cells (Schwartzburd). While HO-1 serves as a potent cytoprotective enzyme against OS, a specific role for HO-1 in this capacity has not yet been established. Experimental evidence stems from observations that cellular resistance to OS correlates positively with levels of HO-1 expression, and cells derived from HO-1-deficient mice are highly susceptible to the accumulation of ROR and oxidative injury (Poss). The cytoprotective feature of HO-1 may be conferred by bilirubin, the end-product of heme degradation known to be an important cellular antioxidant.

Immunohistochemical studies of AD brains have shown that intracellular levels of antioxidant enzymes, namely HO-1, increased several fold in microglial cells adjacent to NFT and senile plaques (Pappolla; Smith, 1994b). Furthermore, the spatial distribution of HO-1 expression in AD brains was found to be essentially identical to that of the pathogenic conformational changes of tau protein, the major component of NFT (Takeda). Similarly, experiments in mice undergoing AA amyloidosis, after injection with AEF/silver nitrate, demonstrated ~3-fold upregulation of HO-1 expression in splenic monocytoid cells, resulting from the activation of the NF-κB transcription factor through engagement of the RAGE receptor (Yan). In this model of AA amyloidosis, AEF/silver
nitrate induces amyloid deposition within days of injection and mice are killed after 5 days of treatment (Yan). On the other hand, the AHC-mouse model, with mice sacrificed at different time periods p.i. for up to 12 weeks, allows the immunohistochemical study of the spatio-temporal distribution of HO-1 expression with respect to both SAA and AA fibril depositions. The results of this study, along with in vitro experiments to ascertain the possible trigger for HO-1 expression in monocyteid cells, are presented in the following chapter.
CHAPTER 2.

HEME-OXYGENASE-1 RESPONSE, A MARKER OF OXIDATIVE STRESS, IN A MOUSE MODEL OF AA AMYLOIDOSIS

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Key words: alveolar hydatid cyst, heme-oxygenase, oxidative stress, AA amyloid, serum amyloid A, image analysis, western blotting, immunohistochemistry.

Abbreviations: HO-1 = heme-oxygenase-1; SAA = serum amyloid A; R-AA = rabbit anti-mouse AA amyloid IgG; R-HO-1 = rabbit anti-mouse HO-1 IgG; SF = splenic follicle; PFZ = perifollicular zone; RP = red pulp; AHC = alveolar hydatid cyst; RE cells = reticuloendothelial cells; mΦ = macrophages; ROS = reactive oxygen species; Ox-St = oxidative stress.

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ABSTRACT

Expression of heme-oxygenase-1 (HO-1), an important marker of oxidative stress, has been studied extensively in the context of Alzheimer’s disease. Evidence of HO-1 expression during AA amyloidosis is, at best, sketchy. Here we present comparative data on HO-1 response in alveolar hydatid cyst (AHC) infected amyloid sensitive (C57BL/6) and amyloid resistant (CE/J) mouse strains. Histochemical and peroxidase-immunoperoxidase methods were used to monitor serum amyloid A (SAA) and AA fibril deposition and HO-1 expression in hepato-splenic reticuloendothelial (RE) cells of the AHC-infected mice prior and during AA fibril deposition. Based on the cumulative data, we conclude that HO-1 expression corresponded closely with tissue deposition of SAA, but was unrelated to AA fibril deposition. To ascertain whether SAA deposition might act as the trigger for HO-1 expression in the RE cells, macrophages were incubated for up to 72 hr with SAA-containing mouse serum. The SAA-treated macrophages, although negative for HO-1 protein, demonstrated SAA in the cell extracts and immunocytochemically in the vacuolar compartments, indicating macrophage-mediated endocytosis and trafficking of SAA. In sum, these results exclude SAA and AA fibrils as the primary triggers in the induction of HO-1 expression in RE cells; the roles of inflammatory cytokines in this process need to be investigated further.
INTRODUCTION

Amyloid-related diseases are characterized by extracellular deposition of non-branching insoluble protein fibrils, called amyloid, in various soft organs\textsuperscript{1,2}. The in vivo mechanism of amyloid fibril formation, however, remains obscure. To understand this mechanism, we are using a parasite (alveolar hydatid cyst, AHC)-mouse model of reactive AA amyloidosis\textsuperscript{3}. AHC grows like a solid tumor in mice, induces chronic inflammation, a significant increase in serum amyloid A (SAA), the precursor of amyloid A (AA), and multi-organ AA fibril deposition starting at about 1 week post-infection (p.i.)\textsuperscript{4,5}. Thus, AHC is a potent inducer of chronic inflammation, SAA synthesis and AA amyloidosis\textsuperscript{4-6}.

Given that chronic inflammation and overproduction of acute-phase SAA are central to the pathogenesis of AA amyloidosis, we and others have proposed that reactive oxygen species (ROS) generated by activated macrophages (m\textsuperscript{\(\Phi\)}) and reticuloendothelial (RE) cells, which are also intimately involved in SAA clearance, could initiate intralysosomal nascent AA fibril formation\textsuperscript{6-9}. Interestingly, oxidative stress (OX-St) and/or low pH conditions have been ascribed to the generation of amyloid-like fibrils from both amyloidogenic and non-amyloidogenic proteins\textsuperscript{10-13}. More specifically, the role of OX-St response has been studied extensively in the pathogenesis of Alzheimer's disease (AD)\textsuperscript{14-16}. As such, heme-oxygenase (HO)-1 and several other OX-St markers were localized to neurofibrillary tangles, around senile plaques, and in neuronal cells\textsuperscript{17,18}. Takahashi et al. recently showed intracellular protein-protein interaction in the endoplasmic reticulum between Alzheimer amyloid precursor protein and HO-2\textsuperscript{19}. Such an interaction was
considered to modulate the protective function of HO, cause exacerbation of oxidative stress and augmentation of neuronal cell death.

HO is a stress protein and is expressed by different cell types including monocytoid cells\(^{20}\). It functions as the initial and rate-limiting step in toxic heme degradation into biliverdin\(^{21}\). Of the three HO isoforms, HO-2 and HO-3 expressions are constitutive but that of HO-1 (aka HSP-32) is inducible at the transcriptional level and is tissue-dependent\(^{22}\). It is found at relatively higher levels in splenic tissues where pro-oxidant heme is degraded\(^{21}\). HO-1 expression is also up-regulated in response to heat shock and inflammatory cytokines and during OX-St by NFκB and AP-1 transcription factors\(^{21-23}\).

OX-St response is a corollary of chronic inflammation that is considered to be a key factor in the pathogenesis of AA amyloidosis. Whether an OX-St-related factor plays any significant role in the disease process, is yet to emerge\(^{24-26}\). Here we present the profiles of HO-1 expression, a marker of OX-St response, in both splenic and hepatic RE cells in AA amyloid susceptible (C57BL/6) and AA amyloid resistant (CE/J) mouse strains and compare the levels of HO-1 expression with tissue deposition of SAA and AA fibrils. Our results indicate that tissue deposition of SAA corresponds with elevated HO-1 expression in the RE cells prior to AA deposition in the AHC-infected C57BL/6 mice; data from AA amyloid resistant CE/J mice support this conclusion. Furthermore, our results from in vitro experiments suggest that SAA alone does not induce HO-1 response in mΦ suggesting that other factors including inflammatory cytokines may have a primary role in oxidative stress response.
MATERIALS AND METHODS

Infection

Six-week-old male C57BL/6 and CE/J mice (Jackson Laboratories, Bar Harbor, Maine) were inoculated intraperitoneally with 250 AHCs, the larval stage of Echinococcus multilocularis. The methods of inoculum preparation and infection and the maintenance of AHC in our laboratory have been published elsewhere. Three to six mice were sacrificed at various time periods between 3 days and 12 wk p.i. Peritoneal AHC masses and spleens were harvested from each mouse and weighed. Portions of spleens and livers were sectioned (6-8 µm thick) using a cryostat and stored at -20°C until used. Control samples were obtained from non-infected mice of matching strain, sex and age.

Immuno- and histochemistry

The tissue sections were stained with Congo red. Skip or adjacent spleen/liver sections were stained using biotin-strepavidin-peroxidase method. Rabbit anti mouse-AA amyloid IgG, (R-AA 1.24 mg/mL; working dilution 1:200 or 1:400), rabbit anti-HO-1 IgG (R-HO-1SPA-896; StressGen Biotechnologies Co., Victoria, Canada; working dilution 1:1000) and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2 dilution, DAKO EnVision+), were used to localize AA fibrils/SAA and HO-1. Following immunostaining, the sections were stained lightly (10 secs) with Harris hematoxylin (Sigma Diagnostics, St. Louis, MO), counterstained with thioflavin S (1% in distilled water, 5 min), dehydrated and mounted with permount. SAA-treated mΦ (see below),
permeabilized with 2% Triton X-100, were immuno-stained for SAA and HO-1, as described 6.

To determine specificity of immunostaining, control sections were treated either with the buffer alone or absorbed R-AA/R-HO-1. Purified mouse AA amyloid and HO-1 (SPT-896, Stressgen Biotechnologies Co.) were incubated with the primary antibodies in a ratio of 20:1 (w/w) for 30 min at 37°C and then overnight at 4°C. The treated antibodies were centrifuged at 4°C for 30 min and the supernatants used for immunostaining.

Image analysis

Image analysis was used to quantify concentration of R-HO-1 RE cells in the spleen and liver sections. The sections were digitized using a microscope (Leitz Dialux 20) and a camera (Panasonic WV1550 TV camera), which was connected to a computer through a video card. This technique provided grey-scale digitized images where an image is a two-dimensional array of pixels. Each pixel had a value representing its grey-scale intensity. To tabulate the amount of immunostaining within a given section, a two-level thresholding technique was used on the digital images. This technique yielded a black and white image, separating stained from non-stained pixels. The user defined upper and lower threshold values. Pixels with intensity between the upper and lower thresholds were set to zero (black); these were considered the stained pixels. The rest of the pixels were set to 1 (white). To normalize the computed numbers, the user had to find the best upper and lower thresholds with trial and error and by comparing the results with the original image. Once the image was thresholded, the number of stained pixels was counted and divided by the total number of image pixels. This provided the user with the
percentage of stained pixels. The commands for the computation of the percentage area of a tissue section occupied by specific staining were written in Matlab®.

Statistical evaluation for the data was performed by the unpaired t-test. A $P$-value $<0.05$ was taken to be statistically significant.

**Determination of serum SAA concentration**

Retired CD1 mice (Charles River, St-Constant, Canada) were injected subcutaneously with 0.5 ml of 2% AgNO$_3$. Serum was collected after 18 hr, pooled, and its SAA concentration was determined using ELISA, as described $^6$. Briefly, purified murine AA amyloid, starting at a concentration of 50ng/50μl, was used as the standard to measure SAA concentration. The pooled serum contained 1.06 mg/ml of SAA.

**SAA uptake by peritoneal mΦ and RAW264.7 cells**

Peritoneal cells were collected from 6 wk old CD1 mice (Charles River, St-Constant, Canada) and incubated with DMEM for 2 hr to purify peritoneal mΦ by adherence. Peritoneal mΦ and RAW264.7 cells (kindly provided by Dr A. Descôteaux, INRS-Institut Armand Frappier, Université du Québec, Laval, Quebec) were cultured at 37°C with 5% CO$_2$ in W-DMEM (DMEM high glucose containing 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin, Gibco, Burlington, Canada). Three million cells were plated in a 25 cm$^2$ culture flask and treated with 5 ml W-DMEM containing 10% SAA serum. At 12, 24 or 72 hr, the cells were trypsinized to remove SAA bound to the plasma membrane and harvested using a 1mM EDTA solution. Aliquots of mΦ, 500,000 cells, were cytocentrifugated onto glass slides and immunostained for the localization of SAA. The remaining cells were mixed with ice-cold 2x sample buffer containing 6M urea (50 μl of 2x sample buffer / 1 x 10$^6$ mΦ),
boiled (5 min), centrifuged (10,000 RPM, 30 min), and the protein extract equivalent to 500,000 cells/lane was used for fractionation and Western blotting.

**Western Blotting**

Selected C57BL/6, CE/J serum samples and SAA-treated mΦ lysates were used to detect SAA by Western blotting, as described\textsuperscript{30,31}. Briefly, the samples were separated on 12% SDS-PAGE gels containing 6M urea, transferred onto a nitrocellulose membrane and the membrane treated with R-AA (1.24 mg/ml; diluted 1:3000); enhanced chemiluminescence kit (Amersham, Baie d’Urfé, Canada) was used for the detection of SAA band.
RESULTS

*C57BL/6 mice: Cyst and spleen weights post-infection*

As described previously, splenomegaly correlated with increasing cyst weights during the course of the infection (Table 1). Between 1 and 12 wk p.i., there was a ~5-fold increase in the mean spleen weight while the mean cyst weight showed a 48-fold increase.

*C57BL/6 mice: Pattern of SAA, AA and HO-1 responses*

The morphology and the cell composition of the three splenic compartments, i.e. red pulp (RP), perifollicular zone (PFZ) and splenic follicle (SF), including increased numbers of megakaryocytes in the RP in the AHC-mice have been described previously. Briefly, SF in the normal mice were devoid of germinal centers and the RP contained rare megakaryocytes. No R-AA immunoreactivity was observed in normal spleen and liver sections indicating absence of detectable SAA deposition. By contrast, significant numbers of R-HO-1 reactive RE cells were detected in the RP of 6 of 6 normal spleen sections (Fig. 2a); HO-1 positive cells occupied approximately 2% of the splenic tissues (Fig. 1a). The immunostaining was specific; absorbed R-HO-1-, or TBS-treated spleen sections failed to show any HO-1 reactivity. Hepatocytes lacked HO-1 reactivity but a few Kupffer cells in the liver sections did stain weakly for HO-1.

At 3 and 5 days p.i., none of the spleen sections displayed Congo red or R-AA reactive AA fibrils. However, homogeneous R-AA reactivity was localized to the PFZ in 3/5 spleen sections indicating SAA deposition. Figures 2b and 2c show the high and low power view, respectively, of global R-AA immunostaining in the PFZ interstitium.
and within the PFZ cells. Interestingly, R-HO-1 reactive RE cell numbers increased in those spleen sections that demonstrated R-AA reactivity in the PFZ. At 5 days p.i., the RP contained the majority of the R-HO-1 reactive RE cells (Fig. 2d). Figure 1a depicts the global profile of R-HO-1 reactive splenic RE cells before and during AA fibril deposition; the number of R-HO-1 positive cells peaked between 3 and 4 wk p.i., and then declined due to the replacement of the R-HO-1 positive RE cells with increasing load of AA fibril deposition (see below).

At 1 wk p.i., the spleen sections, although negative for Congo red staining, displayed stronger R-AA reactivity in the PFZ areas (Table 1), similar to that shown in figure 2c. In high power view, the PFZ cells displayed punctate cytoplasmic R-AA-reactivity, indicating, as shown previously,\(^6,31\), vacuolar localization of SAA\(^32\). R-AA reactivity was also localized around the central arteriole including its endothelium\(^34\). At this time period, R-HO-1 positive RE cells increased in number in both the RP and PFZ (Fig. 1a).

At 2 wk p.i., the PFZ sinus walls showed segmental AA fibril deposition (Table 1; Fig. 2e), but still contained intact HO-1 positive endothelial cells. The RP had expanded, contained heavy megakaryocyte cell infiltration and scant focal thioflavin S (Fig. 2h) or R-AA positive-AA fibrils (Table 1, Fig. 2f). The RP in the double labeled spleen sections showed relatively high numbers of R-HO-1 positive RE cells (Fig. 2g), especially around the thioflavin AA positive foci (Fig. 2h). The liver sections, although negative for AA deposition at 2 wk p.i., demonstrated strong R-AA reactivity localized to the hepatic sinuses and perivascularly around the central veins and the periportal areas. Kupffer cells labeled strongly with R-HO-1.
Between 3 and 4 wk p.i., AA load had increased in the PFZ and to some extent in the RP (Table 1). This corresponded with reduced numbers of R-HO-1 reactive RE cells in the splenic parenchyma (Fig. 1a). As described previously, liver sections, double labeled with thioflavin S and R-AA demonstrated scanty thioflavin S and R-AA reactive AA fibril in the walls of hepatic blood vessels and hepatic sinuses. However, the majority of R-AA reactive hepatic sinuses lacked thioflavin S staining, indicating sequestration of SAA by the sinus Kupffer cells. Such sinuses also reacted with R-HO-1 (Fig. 2k).

Between 6 and 12 wk p.i., the AA load increased progressively in both the splenic and hepatic parenchyma and this corresponded with a decrease in the number of R-HO-1 positive RE cells (Figs. 1a,b). At 12 wk p.i., almost all the PFZ and a major portion of the RP were infiltrated by AA fibrils. Consequently such an alteration corresponded with a precipitous decrease in the number of R-HO-1 positive RE cells (Figs. 2i,j).

Since the lag period between SAA and AA fibril deposition is relatively short in C57BL/6 mice, it was unclear whether the observed increased HO-1 expression in both the splenic and hepatic tissues reflected AHC infection-induced inflammatory stress or a consequence of tissue deposition of SAA or AA fibril. To obtain insights into these questions, we examined HO-1 response in AHC-infected CE/J mice. These mice do not develop AA amyloidosis but show elevated circulating non-amyloidogenic SAA in response to inflammatory stimuli.

**AHC-infected CE/J mice: pattern of SAA and HO-1 response**

The CE/J mice examined at different time periods p.i. yielded much greater cyst masses. The mean cyst weights at 4, 8 and 12 wk p.i. were, respectively, 1.4-fold, 3.5-fold and 2.4-fold greater than those of the C57BL/6 mice. Regardless of relatively large
cyst weights, the CE/J mice did not show AA fibril deposition. However, between 2 and 12 wk p.i., increasing amounts of R-AA reactivity, similar to that shown in Fig. 2c, was detected in the PFZ including the PFZ cells. Similarly, strong R-AA reactivity was seen in the Kupffer cells lining the hepatic sinuses (not shown). Figures 1a and 1b compare the splenic and hepatic profiles of R-HO-1 reactive RE cells in the AHC-infected C57BL/6 and CE/J mouse strains. Little or no detectable HO-1 reactivity was found in five of five normal CE/J spleen and liver sections (Fig. 2l). In sharp contrast, HO-1 expression peaked at 2 wk p.i., in both the splenic and hepatic tissues in both C57BL and CE/J mice (Figs. 1a,b). As shown in figures 2m and 2n, this reactivity was confined to the RP and persisted at high levels throughout the course of infection in the CE/J mice. As such, even at 12 wk p.i., the SF remained intact in the CE/J mice (compare Fig. 2n with 2i). This can clearly be ascribed to the absence of AA fibril deposition in the CE/J mice.

Effect of acute phase SAA (A-SAA) on HO-1 response in cultured macrophages

A-SAA is known to have proinflammatory properties\(^{24,37,38}\). Since the levels of HO-1 expression followed closely with SAA deposition in both splenic and hepatic tissues in C57BL/6 and CE/J mice, we investigated whether these in vivo findings, as a manifestation of oxidative stress, could be replicated in vitro. As such, we cultured mouse resident peritoneal mΦ and RAW 264.7 cells for 72 hr in DMEM containing 10% A-SAA serum. The cells were immunostained with R-AA and R-HO-1 at 12, 24 and 72 hr (findings for 12 and 24 hr incubations similar, thus not shown). At each time point, each cell type demonstrated vacuolar R-AA reactivity (Figs. 2o,p; only the 72 hr profile shown here); R-HO-1 reactivity was singularly absent. Western blot analysis of the cell extracts, showing \(~12\) kDa R-AA immunoreactive band in the cell lysates, further confirmed
intimate interaction between A-SAA and the macrophages, i.e. endocytosis of A-SAA (Fig. 3).

Detection of SAA in sera from AHC-infected C57BL/6 and CE/J mice

To confirm tissue (splenic and hepatic) deposition of circulating SAA in the AHC-infected C57BL/6 and CE/J mice, we immunoblotted selected serum samples using R-AA. Each C57BL/6 sera (2 μL/lane), obtained at days 3 and 5 and at 1 and 2 weeks demonstrated SAA similar in position to that present in the control silver nitrate stimulated mouse serum (Fig. 4a). Similar, although weaker, SAA reactive bands were found in the CE/J mouse sera (3 μL/lane), obtained at 2, 4, 6 and 8 wk p.i. (Fig. 4b).
DISCUSSION

The principal finding in this study was that AHC infection induced significantly increased expression of HO-1 in RE cells of both amyloid-sensitive C57BL/6 and amyloid-resistant CE/J mice. The factor(s) stimulating the HO-1 response in this model and its overall implication in AA amyloidosis are under study.

On an immunocytochemical basis, HO-1 positive splenic and hepatic RE cells increased numerically in both mouse strains (Figs. 1a,b), and this event coincided with SAA deposition in the spleen (Figs. 2b,c), as well as in the liver. SAA was localized, as shown previously, both interstitially in the tissues examined and also to the vacuolar compartments, most likely to the lysosomes, in the RE cells. This indicated an intimate interaction between SAA and the RE cells. Thus, increased HO-1 expression in the RE cells might tentatively be linked to SAA deposition in the splenic/hepatic tissues. However, despite a similar tissue SAA deposition pattern in the two mouse strains, their HO-1 profile levels, as shown in figure 1a, contrasted significantly, especially between 4 and 12 wk p.i., during the AA fibril deposition phase in C57BL/6 mice. Clearly, this precipitous decline in the number of splenic HO-1 positive cells coincided with tissue displacement by the increasing AA load (Table 1; Fig. 1a; Figs. 2i,j). Thus, it is unlikely that AA fibrils participate directly in increased HO-1 expression in the splenic RE cells.

We found a similar relationship in the hepatic tissues as well (Fig. 1b); increased HO-1 expression in the Kupffer cells appeared to be unrelated to AA fibril deposition. Amyloid-resistant AHC-infected CE/J mice unambiguously confirmed this relationship (Figs. 1a,b; Figs. 2m,n); HO-1 response in CE/J mice was more pronounced and
sustained in the absence of AA fibril deposition. It is also worth noting that the pattern of
HO-1 response described here (Figs. 1a,b) was reminiscent of increased ubiquitin (a
stress protein) expression in mΦ/RE cells in the AHC-infected C57BL/6 mice. We had
proposed AHC-infection induced inflammatory stress to be the inducing factor for the
ubiquitin response\textsuperscript{31}.

Recently, Yan et al. showed that both AA fibrils and amyloidogenic murine
SAA1.1, but not SAA2.1 or SAA2.2 (CE/J mouse derived), both of which are non-
amyloidogenic, bound to murine BV-2 cells, a transformed mouse microglial line. These
ligands bound to the BV-2 cells through the receptor for advanced glycation end-products
(RAGE), and induced increased expression of HO-1 mRNA, HO-1 protein and several
inflammatory cytokines\textsuperscript{24}. However, studies carried out in vitro and at variance with that
of Yan et al., showed that non-amyloidogenic SAA2.2 binds mouse peritoneal mΦ more
avidly than SAA1.1\textsuperscript{39}. Presumably, the apparent difference in the binding affinities of
SAA1.1 and SAA 2.2 to mouse mΦ and BV-2 cells could be due to the fact that two
different cell types were used in these studies; BV-2 cells and murine mΦ could have
different affinities for different murine SAA isoforms. While the in vivo metabolism of
SAA2.2 in CE/J mice has not been as well characterized as SAA1.1/SAA2.1\textsuperscript{40}, our data,
nonetheless, clearly show SAA2.2 deposition in both the splenic and hepatic tissues of
AHC-infected CE/J mice. Such a relationship might suggest interaction between SAA2.2
and the RE cells in situ, a phenomenon similar to that established in the clearance of
SAA1.1/SAA2.1 from the circulation\textsuperscript{40}. Thus, the pronounced increase in HO-1
expression in both the splenic and hepatic RE cells of C57BL/6 mice closely correlated
with increased tissue deposition of SAA and independent of AA fibril deposition. The
validity of this argument is strengthened by the data from the AHC-infected CE/J mice. While stress effects are highly complex and multifactorial, we at this stage are unable to single out whether SAA alone or in conjunction with inflammatory cytokines, as would be expected in the AHC-mouse model, effected the induction of HO-1 in the RE cells.

SAA is a sensitive indicator of various tissue insults and inflammatory disorders during which its plasma concentration can increase up to 1000-fold. Clearance of circulating SAA is known to be mediated through activated mΦ/RE cells and lysosomes appear to participate in SAA processing. In view of the fact SAA manifests proinflammatory properties, its interaction with monocytoid cells could then explain the induction of HO-1 response. The data derived from the AHC-infected mice would tend to support this observation (Figs. 1a,b). However, the in vitro experiment performed with mΦ and SAA-containing mouse serum clearly showed that, despite SAA endocytosis, the mΦ failed to show HO-1 response (Figs. 2o,p and Fig. 3). Thus, it is quite likely that multiple factors generated during chronic inflammation in the AHC-infected mice, such as inflammatory cytokines and/or oxidative stress, could have triggered HO-1 expression in the RE cells as a cytoprotective mechanism.
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**TABLE 1.** Mean spleen and cyst weights in normal and alveolar hydatid cyst infected C57BL/6 mice at various times post-infection (p.i.); also shown here is the pattern of serum amyloid A (SAA) and amyloid A (AA) fibril deposition in the splenic perifollicular zones (PFZ) and the red pulp (RP).

<table>
<thead>
<tr>
<th>Time p.i.</th>
<th>Mean weights ± sd Cyst (g)</th>
<th>Mean weights ± sd Spleen (mg)</th>
<th>AA distribution in spleen AA in PFZ</th>
<th>AA distribution in spleen AA in RP</th>
<th>SAA deposition in the spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>-</td>
<td>81.3±6</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 wk p.i.</td>
<td>0.1</td>
<td>85±5</td>
<td>-</td>
<td>-</td>
<td>+*</td>
</tr>
<tr>
<td>2 wk p.i.</td>
<td>0.17±0.04</td>
<td>112±7</td>
<td>2+</td>
<td>1+**</td>
<td>ID†</td>
</tr>
<tr>
<td>4 wk p.i.</td>
<td>0.46±0.1</td>
<td>147±9</td>
<td>3+</td>
<td>1+</td>
<td>ID</td>
</tr>
<tr>
<td>6 wk p.i.</td>
<td>0.53±0.07</td>
<td>192±5</td>
<td>3+</td>
<td>3+</td>
<td>ID</td>
</tr>
<tr>
<td>8 wk p.i.</td>
<td>1.9±0.2</td>
<td>235±17</td>
<td>4+</td>
<td>4+</td>
<td>ID</td>
</tr>
<tr>
<td>12 wk p.i.</td>
<td>4.8±0.9</td>
<td>390±10</td>
<td>4+</td>
<td>4+</td>
<td>ID</td>
</tr>
</tbody>
</table>

* +, homogeneous non-fibrillar rabbit anti-mouse AA amyloid IgG (R-AA) reactivity, indicative of serum amyloid A (SAA) deposition, in the PFZ and the RP.

** Low 1+ to heavy 4+ AA fibril deposition in the PFZ sinuses and the RP

† R-AA immunopositive loci present in the PFZ and the RP but indiscernible (ID) whether SAA or AA fibrils.
FIGURES

FIGURE 1a: Mean percentage (±sd) of HO-1 positive reticuloendothelial cells occupying the sectional areas in spleens from normal and AHC-infected C57BL/6 and CE/J mice at various time periods post-infection.

FIGURE 1b: Mean percentage (±sd) of HO-1 positive Kupffer cells occupying the sectional areas in livers from normal and AHC-infected C57BL/6 and CE/J mice at various time periods post-infection.
FIGURE 2: Spleen and liver sections from normal and alveolar hydatid cyst-infected C57BL/6 and CE/J mice (a-n) and mouse macrophages (o,p) immunostained with rabbit anti-mouse HO-1 IgG (a,d,g,i,k,l,m,n), rabbit anti-mouse AA IgG (R-AA; b,c,f,o,p) or stained with Congo red (e) or thioflavin S (h,j). a. Spleen section from a normal C57 mouse; note HO-1 positive reticuloendothelial (RE) cells in the red pulp (RP); follicle (F)
essentially negative for HO-1 staining.  

b. Spleen section from a 3 days post-infection (p.i.) C57 mouse; note R-AA reactivity, most likely SAA, in the perifollicular (PFZ) cells and the interstitium.  

c,d. Spleen sections from a 5 days p.i. C57 mouse; note SAA immunostaining in the PFZ and the RP (c) and HO-1 immunostaining mostly in the RP (d).  

e. Spleen section from a 2 wk p.i. C57 mouse; note Congo red positive AA deposits in the PFZ sinus walls.  

f-h. Spleen sections from a 2 wk p.i. C57 mouse; note moderate R-AA immunostaining in the RP (f); double labeling showing HO-1 positive RE cells in the RP (g) around thioflavin S positive AA deposits (h).  

i,j. A double-labeled spleen section from a 12 wk p.i. C57 mouse; note the decrease in HO-1 positive RE cells around the follicle (i) and the surrounding PFZ and the RP showing massive thioflavin S positive AA deposits (j).  

k. Liver section from a 4 wk p.i. C57 mouse; note HO-1 positive hepatic sinuses (S) and central vein endothelium (CV).  

l-n. Spleen sections from normal, 2 wk p.i., and 12 wk p.i. CE/J mice; note residual HO-1 immunostaining in the normal spleen (l), and strong HO-1 immunostaining in the morphologically intact RP at both 2 wk p.i., (m) and 12 wk p.i., (n).  

o,p. Resident peritoneal macrophages from CD1 mouse (n) and RAW264.7 cells (o) cultured with 10% SAA-containing mouse serum for 72 hr and reacted with R-AA; note strong vacuolar SAA immunostaining.  

Original magnification: a,c,e,i,j,l,m,n 10x; d,f,k 16x; b,g,h,o,p 60x.
FIGURE 3: Immunoblot to detect SAA in resident mouse peritoneal macrophages (per. mΦ) and RAW264.7 cell lysates. The cells were cultured for 72 hr in DMEM (5% CO$_2$, 37°C) containing 10% pooled serum from CD1 mice obtained 18 hr after a subcutaneous injection with 0.5 ml of 2% AgNO$_3$. The cells were trysinized, washed with phosphate buffer, lysed and each lane was loaded with clarified cell extracts equivalent to 500,000 cells; rabbit anti-mouse AA amyloid IgG (1.24 mg/mL, dilution 1:3000) and ECL kit were used to develop the immunoblot.
FIGURE 4: Immunoblots, developed separately, to detect serum amyloid A in sera from alveolar hydatid cyst-infected C57BL/6 (2 μL/lane) and CE/J (3 μL/lane) mice at various time periods post-infection (p.i.).

a. C57BL/6 mice: lanes 1-4 contain sera at 3 and 5 days, 1 and 2 wk p.i., respectively; lane 5, AgNO₃-treated CD1 mouse (2 μL/lane).

b. CE/J mice: lane 1, as lane 5 in a.; lane 2-5 contain sera at 2, 4, 6 and 8 wk p.i., respectively; rabbit anti-mouse AA amyloid IgG (1.24 mg/mL, dilution 1:3000) and ECL kit were used to develop the immunoblots.
ACKNOLEDGEMENTS

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PREFACE TO CHAPTER 3

In the AHC-mouse model of AA amyloidosis, HO-1 expression corresponded closely with tissue deposition of SAA, but was unrelated to AA fibril deposition. In vitro studies however, demonstrated that SAA alone was not responsible for the upregulation in HO-1 response in mΦ. Given that chronic inflammation is central to the pathogenesis of AA amyloidosis, the role of inflammatory cytokines and OS must be clarified in order to better understand the dynamics of the observed HO-1 response.

While HO-1 serves solely as a marker of antioxidant response, a cytoprotective response to OS, markers of oxidative modifications explicitly confirm OS and allow for immunohistochemical localization of such modifications. Specific markers against different oxidative modifications allow for the determination of the extent and type of such modifications in tissue, specifically in the fixed and migrating monocytoid cell populations. Immunohistochemical localization of two such oxidative modifications was performed in spleen sections and peritoneal leukocytes from AHC-infected mice; the results are presented in the following chapter.

A variety of oxidative modifications is formed from the effect of ROR. Our study focused on the distribution of markers of lipid peroxidation and advanced glycation end-products, specifically 4-hydroxy-2-nonenal (HNE) and Nε-(Carboxymethyl)lysine (CML), respectively. Both markers have been immunolocalized to chemically diverse amyloid deposits, including human AA fibrils (Ando). Interestingly, a recent AD study has shown that a cross-link modification induced by HNE on tau, responsible for the Alz50 epitope that is prone to NFT formation, is completely coincident with HO-1 expression (Takeda, 2000b).
Chapter 3.

Immunolocalization of lipid peroxidation end products in murine AA amyloidosis

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Running title: Oxidative stress markers in AA amyloidosis

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ABSTRACT

Chronic inflammation, superimposed by amyloid fibril deposition, is believed to trigger the cascade of oxidative stress response in the affected organs and tissues. We examined immunohistochemically the distribution of two markers of oxidative stress, 4-hydroxy-2-nonenal (HNE) and Nε-(carboxymethyl)lysine (CML), in spleen sections and peritoneal macrophages (mΦ) from mice prior to and during AA amyloidosis. With time, both HNE and CML immunoreactivities increased significantly, particularly in mΦ and splenic reticuloendothelial cells, known to be associated with the clearance of serum amyloid A, the precursor of AA fibrils. In addition, the cellular increase in HNE and CML corresponded with the progression of AA fibril deposition in the splenic tissue. HNE and CML were localized to the plasma membrane and the cytoplasmic compartment of mΦ and HNE only at the nuclear membrane. These markers were also co-localized bound to AA fibril infiltrating the splenic sinus walls. Our results reinforce the notion that oxidative stress is an integral component of amyloidotic tissues. Both HNE and CML have been implicated in protein modification and amyloid fibril formation. The significance of HNE and CML associated with the monocytoid cells, implicated in SAA clearance and AA fibril formation, is discussed with the pathogenesis of AA fibrils.

Keywords – AA amyloid, Alveolar hydatid cyst, Oxidative stress, 4-Hydroxy-2-nonenal, Lipid peroxidation, Nε-(carboxymethyl)lysine, Advanced glycation end product, Immunohistochemistry, Splenic perifollicular zone, Red pulp, Reticuloendothelial cells, Macrophage.
INTRODUCTION

Amyloid diseases represent a broad spectrum of ailments characterized by the extracellular deposition of insoluble protein fibrils, originating from a particular precursor protein, in affected soft organs [1]. Involvement of kidney glomureli, infiltrated with AA amyloid, in inflammation-associated AA amyloidosis poses a serious clinical condition in diseases such as rheumatoid arthritis and chronic bacterial and parasitic infections such as tuberculosis and leprosy [2,3]. However, the conditions that lead to transformation of soluble precursor protein, serum amyloid A (SAA), into amyloid A (AA) fibrils are unknown. AA fibrils are 5 to 13 nm in thickness, have cross β-pleated sheet structure, bind Congo red dye and may induce oxidative stress in the tissue monocytoid cells [4,5]. We have used the alveolar hydatid cyst (AHC)-mouse model to understand the mechanisms of AA fibril formation [6]; the course of the disease in mice mimics the course of AA amyloidosis in humans. AHC grows like a solid tumor, inducing chronic inflammation and a robust SAA response, culminating into multiple-organ AA fibril deposition [3,6].

Chronic inflammation has long been recognized as central to the induction of AA amyloidosis, although its role in this process has not yet been fully investigated [2,7]. More specifically, oxidative stress has recently been implicated as a contributing factor to the generation of AA fibrils [5,8]. Recently, we showed heme-oxygenase-1 (HO-1) response in the AHC-mice prior to and during AA amyloidosis and we associated this response to preponderant SAA deposition in the soft organs [9]. In previous studies we showed that SAA is endocytosed by tissue monocytoid cells, trafficked to lysosomes and...
as a result of partial degradation, at least five N-terminally intact SAA derivatives, similar in molecular masses to that of tissue AA fibril protein, are produced [10,11]. This led us to propose that AA fibrils might be generated intracellularly in tissue monocytoid cells. As such, Kluve-Beckerman et al., in a series of publications, confirmed this finding [12,13]. However, the conditions that led to AA fibril synthesis were not discussed. Since multiple mediators of inflammation induce generation of copious amounts of reactive oxygen radicals (ROR) in the monocytoid cells, it is reasonable to propose that these processes may affect the metabolism of reticuloendothelial (RE) cell/macrophage (mΦ)-associated SAA. We hypothesized that ROR generated in inflammatory mΦ/RE cells could oxidize SAA fragments, render these fragments resistant to enzymatic degradation/clearance and prone to AA fibril formation [14]. However, the extent that ROR might affect intracellular nascent AA fibril formation remains to be determined.

Various oxidative modification products are formed from the effect of ROR; protein modifications that occur under conditions of oxidative stress may result from lipid peroxidation and/or glycoxidation [15]. Lipid peroxidation, originating in biological membranes by the cleavage of oxidized unsaturated fatty acids of cholesterols and phospholipid esters, generates a complex pattern of aldehydes of which 4-hydroxy-2-nonenal (HNE) is one of the major reactive products [16]. Glycoxidation of proteins by reducing sugars produces advanced glycation end products (AGEs), usually a slow process, moderated by the presence of oxygen [17]. N\textsuperscript{ε}-(Carboxymethyl)lysine (CML), a glycoxidation product, can also be formed independently through lipid peroxidation [18]. Both markers have been localized to chemically diverse amyloid deposits: HNE
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immunoreactivity (IR) was localized to amyloid positive blood vessel walls and mΦ around amyloid deposits in patients with primary and secondary amyloidosis [8], and to β-amyloid in Alzheimer’s disease [19,20]; CML-IR was localized to AA fibrils in kidney glomeruli [21,22], β2-microglobulin amyloid fibrils in patients with long-term dialysis [17], and to Alzheimer’s β-amyloid [23,24]. These two markers showing HNE-IR and CML-IR have been co-localized in amyloidotic tissues of patients with uremia [25,26] and Alzheimer’s disease [27]. Modification of these proteins through HNE and CML could render them resistant to proteolytic degradation and present them as tombstone markers. It has recently been proposed that extensive protein oxidation may render such aggregates highly resistant to proteolysis [28,29].

The aim of our investigation was to immunohistochemically monitor the spatio-temporal distribution of HNE-, and CML-IR in the AHC-mice, prior to and during AA fibril deposition. HNE and CML were co-localized to peritoneal mΦ, splenic RE cells and interstitium, and AA fibrils. These data support our previous findings showing stress response in the AHC-mice undergoing AA amyloidosis [9,30].
MATERIALS AND METHODS

Infection

In our previous study, we used spleen and liver sections from the AHC-mouse to examine the profile of HO-1 response [9]. Tissue sections from the same series of experiments were used in the present study. Briefly, six-week-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) were inoculated intraperitoneally with 250 alveolar hydatid cysts. Three to six mice were sacrificed between 5 days and 12 wk post infection (p.i.); cryostat spleen sections (6-8 μm thick) and cytaceentrifuged peritoneal leukocytes were used for immunostaining. Control samples were obtained from non-infected C57BL/6 mice.

Antibodies

Commercially available rabbit anti-4-hydroxy-2-nonenal serum (R-HNE, Cat # HNE11-S, Alpha Diagnostic International, San Antonio, TX) and rabbit anti-carboxymethyllysine serum (R-CML, a generous gift from Dr. George Perry, Institute of Pathology, Case Western Reserve University, Cleveland, OH) were used in 1:500 and 1:200 dilutions, respectively, for the detection of lipid peroxidation and advanced glycation end product formation. The secondary antibody was goat anti-rabbit IgG conjugated to peroxidase labeled-dextran polymer (K4002, Dako EnVision+, Dako Corporation, Carpinteria, CA).

Immunohistochemistry

Some modifications were applied to the basic method described previously [30]. Spleen sections and cytaceentrifuged cells were fixed in 4% paraformaldehyde (20 min)
and treated sequentially with the following reagents: 3% H₂O₂ in methanol for quenching endogenous peroxidase activity (30 min), washing 3x in phosphate buffered saline (PBS, pH 7.4), incubation in blocking solution (BS; 0.5% BSA, 0.1% saponin, 2.5% non-fat dry milk, 1% H₂O₂) containing 5% normal rabbit serum (45 min), incubation in primary antibody diluted in BS (overnight), washing 3x in BS, incubation in secondary antibody diluted in BS containing 5% normal goat serum (1 hr), and washing 3x in PBS. Color reaction was developed with diaminobenzidine (up to 15 min) and the samples were then lightly counterstained with hematoxylin (Harris’ formulation, Sigma). Double-labeling of the immunostained spleen sections were carried out with thioflavin S (1% in distilled water, 5 min). Specificity of the immunostaining was performed by incubating the samples in PBS without the primary antibody or in absorbed R-HNE antibody; absorption was carried out by overnight incubation of 0.1 ml of R-HNE with 0.1mg of HNE-KLH peptide, kindly provided by Dr. George Perry. The absorbed antibody was microcentrifuged and the supernatant used instead of R-HNE. Digital pictures were taken using a Nikon 100 camera mounted on a Leitz Dialux 20 microscope.

Between 300 to 400 immunostained cells were examined at each time period, under 60x (oil immersion); data is presented as the mean of immunopositive cells ± sd.
RESULTS

Cyst and spleen weights and immunohistochemical localization of SAA and AA fibrils in spleen sections

The data on splenomegaly and peritoneal AHC masses, harvested from mice at different time post-infection, have been published [9]. Briefly, splenomegaly corresponded with progressive increase in the peritoneal AHC-biomass and peritonitis [31]. Between 1 and 12 wk p.i., there was a 5-fold increase in the mean spleen weight and a 48-fold increase in the mean AHC weight.

In order to connect the present data with the profiles of SAA and AA fibril deposition in the splenic tissue, we briefly summarize here our previous findings [9]. Normal spleen sections lacked IR against rabbit anti-mouse AA amyloid antibody (R-AA) which immunoreacts with both SAA and AA amyloid. At 5 days p.i., the spleen sections were devoid of AA fibril deposition, but they did demonstrate homogeneous R-AA-IR sites at the perifollicular zones (PFZ) indicating commencement of SAA deposition in the tissue. At 1 wk p.i., spleen sections lacking Congo red staining, displayed stronger punctate R-AA-IR in RE cells in the PFZ. Between 2 and 12 wk p.i., progressive AA fibril deposition caused radical architectural changes in the splenic parenchyma. Endocytosed SAA with punctate R-AA-IR in the peritoneal mΦ was detected at 5 days and onwards [32]. In sum, SAA was detected in the splenic tissue including the tissue mΦ 1 wk prior to AA fibril deposition.
Immunohistochemical localization of HNE in peritoneal leukocytes

Between 1 to 12 wk p.i., mΦ constituted the predominant population. Fig. 1 shows the mean ± sd of mΦ with plasma membrane/cytoplasmic-IR. Between 1 and 6 wk p.i., the number of mΦ with HNE-IR increased ~3-fold over the normal cells, i.e. from 30% to 80%; the pattern of HNE-IR in cells, whether membranous or cytoplasmic, varied. After the peak response at 6 wk p.i., the number of mΦ with HNE-IR decreased gradually to less than 50% by 12 wk p.i. This may indicate freshly recruited mΦ to the peritoneal cavity. Only a fraction of normal mΦ displayed light membrane HNE-IR (fig. 1, fig. 2A).

The spatial distribution of HNE-IR, as shown in fig. 1, varied with time and the progression of AHC-infection. At 1 wk p.i., ~20% of the mΦ displayed a single dot-like cytoplasmic HNE-IR site (fig. 2B); the IR site was smaller than a nucleus but much larger than a lysosome (> 1 μm). Between 2 and 6 wk p.i., the initial site of HNE-IR at the membrane extended to the cytosolic compartment; nuclear HNE-IR was rarely seen at these time periods. At 8 and 12 wk p.i., while the number of mΦ with HNE-IR decreased gradually, the positive cells displayed multiple focal HNE-IR in the cytoplasm as well as relatively intense HNE-IR in both the plasma and nuclear membranes (fig. 2C,D).

Immunohistochemical localization of HNE in spleen sections

The profiles of HNE-IR localized to three splenic compartments, i.e. splenic follicle (SF), red pulp (RP) and PFZ, are described here. Normal spleen sections displayed focal granular interstitial HNE-IR in the RP (fig. 2E,F). As described
previously, the normal spleen sections lacked R-AA-IR, indicating lack of SAA or AA fibril deposition [9].

At 5 days and 1 wk p.i., spleen sections showed variable HNE-IR, ranging from light to relatively intense, confined to both the splenic interstitium and the RE cells in the RP (fig. 2G). In high power view, HNE-IR monocytoid cells appeared to abut on the follicle indicating HNE-IR in the PFZ cells (fig. 2H). Occasionally, follicular central arteriolar endothelium showed intense HNE-IR (fig. 2I).

At 2 weeks p.i., the RP had expanded and the PFZ sinus walls were infiltrated with thioflavin S positive AA fibrils (fig. 2J). Large clusters of RE cells in the RP showed strong HNE-IR, adjacent to amyloidotic PFZ (fig. 2K). In addition, lumens of both RP and PFZ sinuses contained diffuse HNE-IR that was also localized to the inner margin of the infiltrated AA-fibril deposits (fig. 2L_M).

Between 3 and 12 wk p.i., the HNE-IR was global throughout the splenic tissues including the RE cells in the follicles; HNE-IR to the inner margin of AA-fibrils in the sinusoids persisted (fig. 2N). By 12 wk p.i., massive infiltration of AA fibrils to both the RP and the PFZ had diminished the cell population in the splenic parenchyma; this led to an overall diminution of HNE-IR.

*Immunohistochemical localization of CML in peritoneal mΦ*

In previous studies, concomitant HNE-, and CML-IR have been localized to amyloid deposits in patients with diverse amyloidoses [25-27]. Finding of robust HNE-IR in both peritoneal mΦ and splenic tissues led us to examine the incidence of CML.
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Figure 3 shows the profile of mΦ with CML-IR; less than 15% of normal mΦ were positive. At 1 wk p.i., there was a ~5-fold increase in the number of mΦ with CML-IR; up to 80% of the cells were positive. Plasma membrane CML-IR was particularly high (fig. 3, fig. 4A). Notably, at this time period, the number of mΦ with CML-IR was ~ 3-fold greater than that with HNE-IR.

Between 2 and 6 wk p.i., the number of mΦ with CML-IR increased to 90%; up to 60% of the cells had punctate cytoplasmic CML-IR (fig. 3, fig. 4B). At 8 wk p.i., the number of positive cells decreased to 60% and the majority of such cells displayed multiple vesicular cytoplasmic CML-IR (fig. 4C). At 12 wk p.i., only ~ 30% of cells displayed CML-IR (fig. 3). Although, with time, the number of mΦ with HNE-IR and CML-IR had increased, their respective IR pattern differed to some extent; CML-IR as compared to HNE-IR was rarely localized to the nuclear membrane (compare fig. 2D to fig. 4C).

Immunohistochemical localization of CML in spleen sections

Both the pattern and incidence of CML-IR in the splenic tissues were similar to those described for HNE-IR. Briefly, the normal spleen sections displayed both interstitial and intracellular CML-IR in the RP (fig. 4D); this reactivity increased at 5 days and some of the positive RE cells appeared to encroach onto the PFZ (Fig. 4E).

At 2 wk p.i., CML-IR was localized to AA fibril deposits in both the PFZ and the RP (fig. 4F). Topographically, CML-IR was similar to that of HNE-IR, that is CML bound to the inner margin of AA fibrils infiltrating the PFZ sinus walls (fig. 4G,H). The lumens of such sinuses were devoid of leukocytes but contained diffuse CML-IR,
indicating release of this product by the dying/dead endothelial cells. Between 3 and 12 wk p.i., as indicated previously, AA fibril deposition increased progressively in the splenic tissue but without any manifest differences in CML-IR (fig. 4I). Notably, at these time periods, both HNE-IR and CML-IR were replica of each other except that the former appeared segmental while the latter was diffuse and uniform (fig. 2N, 4I). Figures 4J and K show co-localization of CML-IR to thioflavin fluorescent AA fibrils.
DISCUSSION

The principal findings, based on immunohistochemical studies, were that both splenic RE cells and peritoneal mΦ from mice undergoing AA amyloidosis generated significantly increased levels of HNE and CML adducts. In addition, each of these adducts were immunolocalized to the splenic interstitium, capillary endothelium and the splenic AA fibril deposits.

Normal mΦ/RE cells showed weak HNE-IR and CML-IR but as early as 1 wk p.i., similar to that of HO-1 response in this mouse model [9], immunoreactivity against both these markers increased remarkably in the monocytoid cells. These events, at least spatio-temporally, appeared to correspond with mΦ/RE cell-mediated clearance of humoral SAA during which humoral SAA is endocytosed, trafficked to lysosomes where it is either degraded completely or partially, yielding amyloidogenic SAA fragments [10,30,32,33]. At this point, we do not know whether SAA per se or other mediators of inflammation triggered the oxidative stress response in the monocytoid cells. In a previous study, interaction of SAA/AA fibrils with monocytoid cells, both in vivo and in vitro, was associated with oxidative stress response [5]. In this context, it is important to note that marked leukocytosis and chronic inflammation characterize AHC infection both in humans and mice [34,35]; sera from mice undergoing AA amyloidosis show marked elevation in serum amyloid A, interferon gamma, tumor necrosis factor alpha and granulocyte-macrophage colony stimulating factor (unpublished).

With time and coincident with tissue AA fibril deposition, both HNE-IR and CML-IR increased in the tissue monocytoid cells (fig. 1, 2B-D,G-M, 3 and 4A-C,F-I).
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However, between 8 and 12 wk p.i., there was a marked numerical reduction in peritoneal mΦ with HNE- and CML-IR (fig. 1, fig. 3), while the remaining positive cells displayed multiple HNE- and CML-IR loci in the cytoplasmic compartment but only HNE-IR at the nuclear membrane (fig. 2C,D, fig. 4C). HNE, an amphipatic molecule and a potent electrophile, is known to enter all cellular structures without encountering biological barriers and react with both proteins and DNA [36]. As such, we were able to show HNE-IR at both the plasma and nuclear membranes, in the cytoplasmic compartment and interstitially bound to the AA fibrils. As to the reason for the precipitous decrease in the number of HNE- and CML-IR positive cells between 8 and 12 wk p.i., we suggest that it may be due to the ongoing intense inflammatory reaction and continuous influx of inflammatory infiltrates at the sites of infection [35,37,38].

Of interest is the finding that both HNE- and CML-IR were localized to the endothelium of the central arteriole starting at 5 days p.i., and onwards (fig. 2I, CML not shown). Similar IR was seen in the walls of splenic sinuses infiltrated with AA fibrils (fig. 2L,M, fig. 4H,I). We suggest that co-localization of HNE- and CML-IR at the endothelium may be related to its dysfunction or degeneration. We have shown flattening of high endothelial cells in the lymphoreticular tissues of the AHC-mice coincident with marked lymphadenitis, splenomegaly and depletion of T cells from the thymus-dependent areas [31,39]. As to similar changes seen in the endothelium of splenic sinus walls it is quite likely that they might be amyloid related events. A solid layer of AA fibrils abutting on the strongly HNE- and CML-IR endothelium suggests such a possibility (fig. 2L,M, fig. 4H,I). Indeed, Rocken et al. were able to show a similar pattern of CML-IR in
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endothelial cells adjacent to AA amyloid deposits found in human and murine kidney, spleen and liver sections [21]. Furthermore, Yan et al. showed much the same relationship between oxidative stress response and AA fibrils in murine and human amyloidotic tissue [5]. They showed both SAA and AA fibrils, by interacting through the receptor for advanced glycation end products (RAGE), up-regulated the expression of HO-1, interleukin-6 and macrophage colony stimulating factor in monocytoid cells [5].

Recently, Miyata et al. coined the term ‘carbonyl stress’ in relation to β2-microglobulin amyloid fibril formation and suggested a possible role of increased accumulation of carbonyl compounds in inducing protein modification by glycoxidation and/or by lipid peroxidation reactions [25,26]. As such, similar to our findings in the AHC-mouse model, Miyata et al. co-localized both HNE-, and CML-IR to the amyloid fibrils [25,26]. Evidence shows that in vivo, CML is not only a product of glycoxidation but also of lipid peroxidation; co-localization of HNE-, and CML-IR in amyloidotic tissues manifesting oxidative stress response would be consistent with their common metabolic origin [15,40]. Thus, the observed elevation in CML-IR in our mouse model undergoing AA fibril deposition could reflect increased lipid peroxidation.

One consequence of protein oxidation, including that of glycoxidation/lipid peroxidation, is the formation of intra-, and inter-molecular protein cross-linking that confer protease resistance and extreme insolubility to the modified protein [28,29,41,42]. AGE accumulation has been documented in AD [23,24,27] and AGE-modified Aβ protein has been shown to accelerate in vitro the aggregation of soluble Aβ peptide into fibrils [43]. HNE can form Michael adducts with compounds bearing thiol groups (Cys)
and amino groups (His, Lys) at position C3 and can undergo Schiff base condensation with Lys residues at the C1 carbonyl [15]. When both these reactions occur within one molecule of HNE, protein cross-linking results. HNE, generated intracellularly, was implicated in the inactivation of lysosomal cathepsins via cross-linking and causing inactivation/functional modification [44]. At this stage, our data do not allow us to directly implicate oxidative stress in amyloid formation. Given that lipid peroxidation/glycoxidation modify proteins, it is conceivable that such events could potentially affect mΦ/RE cell-associated SAA rendering it resistant to lysosomal degradation and prone to AA fibril formation [14].

In sum, with time the tissue IR of both HNE and CML intensified and corresponded with the progression of AA fibril deposition. Our results reinforce the notion that oxidative stress is an integral component of amyloidotic tissues regardless of the chemical nature of amyloid deposits. Further investigation is needed to understand the relationship between oxidative stress-derived factors, defective intracellular clearance of SAA and nascent AA fibril formation.
ACKNOWLEDGEMENTS

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ABREVIATIONS

AA – amyloid A
AGE – advanced glycation end product
AHC – alveolar hydatid cyst
BS – blocking solution
CML - N\textsuperscript{\textsubscript{e}}-(carboxymethyl)lysine
HNE – 4-hydroxy-2-nonenal
HO-1 – heme-oxygenase-1
IR – immunoreactive/immunoreactivity
M\Phi – macrophage
PBS – phosphate buffered saline
PFZ – perifollicular zone
SAA – serum amyloid A
SF – splenic follicle
R-AA – rabbit anti-AA amyloid
R-CML – rabbit anti-carboxymethyllysine
RE – reticuloendothelial
R-HNE – rabbit anti-4-hydroxy-2-nonenal
ROR – reactive oxygen radicals
RP – red pulp
REFERENCES


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Figure 1. Profiles of peritoneal mΦ with 4-hydroxynonenal immunoreactivity (HNE-IR) from normal and alveolar hydatid cyst-infected mice between 1 to 12 wk post-infection (p.i.). The histograms compare the mean (± sd) percentage of mΦ without or with HNE-IR.
Figure 2. Peritoneal macrophages (mΦ; A-D) and spleen sections (E-N) from normal and alveolar hydatid cyst-infected mice immunostained with antiserum to 4-hydroxynonenal (HNE). (A) Normal mΦ display residual plasma membrane HNE-immunoreactivity (IR). (B) At 1 wk post-infection (p.i.), mΦ display focal cytoplasmic HNE-IR. (C,D) At 12 wk...
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p.i., mΦ display HNE-IR at the plasma and nuclear membranes and at multiple cytosolic sites. (E,F) Spleen sections from a normal mouse; both low power (E) and high power (F) views showing HNE-IR confined to the red pulp (RP); note both interstitial and intracellular IR in the reticulendothelial (RE) cells. (G-I) At 5 days p.i., note global view of HNE-IR in the spleen (G), in the PFZ (H), and in central arteriolar endothelium (I). (J-M) At 2 wk p.i., note thioflavin S positive AA fibrils in PFZ and RP sinuses (J), a cluster of HNE-IR positive RE cells in the RP (K), and a double-labeled PFZ sinus showing segmental HNE-IR (L) co-localized to the inner margin of thioflavin S positive AA-fibrils (M). (N) At 3 wk p.i., a global view showing segmental pattern of HNE-IR to the amyloidotic sinus walls in the PFZ and RP; note lack of IR in the splenic follicle (SF).

Original magnification: E,G,J,N 16x; B,H,L,M 40x; A,C,I,K 60x; D,F 100x.
Figure 3. Profiles of peritoneal mΦ with Nε-(carboxymethyl)lysine (CML-IR) from normal and alveolar hydatid cyst-infected mice between 1 to 12 wk post-infection (p.i.). The histograms compare the mean (± sd) percentage of mΦ without or with CML-IR.
Figure 4. Peritoneal macrophages (mΦ; A-C) and spleen sections (D-K) from normal and alveolar hydatid cyst-infected mice immunostained with antiserum to Nε-(carboxymethyl)lysine (CML). (A) At 1 wk p.i., mΦ display both membrane and cytoplasmic CML-immunoreactivity (IR). (B) At 6 wk p.i., mΦ demonstrate membrane as well as punctate cytoplasmic CML-IR. (C) At 8 wk p.i., a high power view of a mΦ showing multiple cytoplasmic vesicular CML-IR. (D) Normal spleen showing scant CML-IR confined to the reticuloendothelial (RE) cells in the red pulp (RP). (E) At 5 days p.i., increased global CML-IR mainly in the RP. (F-H) At 2 wk p.i., CML-IR is localized
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to RE cells in the RP and to AA fibril deposits in the inner margin of PFZ sinus walls (F): a double-labeled PFZ sinus showing co-localization of CML-IR to the thioflavin S positive AA fibril deposits (G,H). (I) At 3 wk p.i., note AA fibril-mediated distortion of splenic architecture and a global view of CML-IR localized to AA fibril infiltrates to the sinus walls. (J,K) At 12 wk p.i., note uniform CML-IR localized to the margin of thioflavin S positive AA fibril deposits in the RP. Original magnification: D,E,F,I 16x; A,B,G,H,J,K 60x; C, 100x.
CHAPTER 4. CONCLUDING REMARKS

Chronic inflammation, which is central to the pathogenesis of AA amyloidosis, is known to instigate OS response. OS-related factors could, in their own right, effect the pathogenesis of AA amyloidosis. Since inflammatory monocyteid cells simultaneously generate ROR and process SAA, it is plausible that oxidative modification of cell-associated SAA occurs, resulting potentially in its structural alteration and AA fibril formation. This study represents the groundwork for this hypothesis by providing unequivocal evidence of OS response in the AHC-infected mouse model of AA amyloidosis.

This work characterized the immunohistochemical distribution of OS-related factors generated in vivo in AHC-infected spleen/liver sections and peritoneal mΦ. HO-1, an antioxidant enzyme whose expression implies both inflammation and OS, was localized to splenic/hepatic SAA deposits, regardless of AA fibril formation. SAA was not the primary trigger of the observed HO-1 response as evidenced by in vitro experiments. Other groups however, have shown that SAA, through activation of RAGE, can activate the NFκB pathway, which in turn upregulates HO-1 expression (Yan).

A significant increase in levels of HNE, a lipid peroxidation adduct, and CML, an advanced glycation end-product (AGE), in AHC-mouse derived splenic RE cells and peritoneal mΦ, known to sequester SAA, was demonstrated. Interestingly, HNE and CML immunoreactivities in peritoneal mΦ were demonstrated within cytoplasmic vesicles, an observation reminiscent of SAA immunoreactivity in lysosomal compartments of the same cells (Chronopoulos). Furthermore, binding of both HNE and CML to extracellular AA fibril deposits was evidenced. From these findings, it can only
be deduced, and not confirmed, whether SAA or AA fibrils are oxidatively modified within mΦ/RE cells.

Both lipid peroxidation and AGE, through the formation of intra- and intermolecular protein cross-linkages, have been implicated in protein modification, resistance to degradation and amyloid fibril formation (Markesbery, Grunne). Thus, defective catabolism of mΦ/RE cell-associated SAA, believed to instigate nascent AA fibril formation intracellularly, could be associated with oxidation of SAA (Ali-Khan, 2002). Future work would necessitate structural studies on SAA and oxidized SAA products. We propose that SAA isolated from AHC-mouse derived mΦ/RE cells should be similar or identical to oxidized recombinant SAA. In the end, the knowledge of the mechanisms underlying amyloid formation is truly the key in developing compounds capable of inhibiting fibril formation, in order to retard the progression of amyloid diseases, notably AD.
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