



Pathological Features of Amyloidosis in Stranded California Sea Lions (*Zalophus californianus*)

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Summary

Amyloidosis was diagnosed in 26 stranded adult California sea lions between 1983 and 2006 by retrospective case analysis. The kidneys (92.3% of animals), blood vessels (80.7%) and thyroid glands (65.4%) were most commonly affected. Macroscopically, affected kidneys were swollen, with pale tan cortices and loss of cortico-medullary differentiation. Amyloid deposits in the kidney were located in the glomeruli, blood vessels, and peritubular interstitium, most prominently in the outer stripe of the medulla. The amyloid deposits were identified as type amyloid A (AA) by potassium permanganate staining and immunolabelling with antibodies against AA protein. Concurrent diseases, including inflammatory processes and genital carcinoma, were common in affected animals. Serum amyloid A concentrations were high (>1200 µg/ml) in six of seven affected sea lions, while the median value in clinically healthy animals was <10 µg/ml. These findings suggest that renal amyloidosis contributes to morbidity and mortality in stranded adult California sea lions.

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Keywords: AA amyloidosis; California sea lion; sea lion; *Zalophus californianus*

Introduction

The term amyloidosis refers to a group of diseases characterized by extracellular deposition of abnormal or improperly folded, fibrillar protein in various tissues. Amyloid deposits, which appear as amorphous hyaline material in sections stained with haematoxylin and eosin (HE), show an affinity for Congo red and exhibit characteristic apple-green birefringence under polarized light after Congo red staining. Ultrastructurally, amyloid is composed of non-branching fibrils ranging from 5.7 to 10 nm in diameter. X-ray crystallography and infra-red spectroscopy show that the fibrils are arranged in cross- β -pleated sheets (DiBartola and Benson, 1989; Abbas 2005).

Amyloid can be classified on the basis of its constituent chemical fibrils (e.g., AL, AA, A β), its distribution

pattern (systemic *vs* localized), or its association with other diseases (primary *vs* secondary) (Merlini and Bellotti, 2003). Familial forms of amyloidosis have been described in Shar Pei dogs and Abyssinian cats (Boyce *et al.*, 1984; DiBartola and Benson, 1989). Reactive systemic AA amyloidosis (“secondary” amyloidosis) is associated with chronic inflammatory diseases, infection, or some forms of cancer. Secondary amyloidosis results from tissue deposition of an N-terminal fragment of serum amyloid A protein (SAA). Serum amyloid A is an acute-phase reactant apolipoprotein synthesized by hepatocytes and histiocytic cells under the influence of inflammatory cytokines such as interleukin (IL)-1, IL-6, and tumour necrosis factor α (DiBartola and Benson, 1989; O’Hara *et al.*, 2000). High concentrations of SAA, consistent with an acute phase protein response, have been reported in human beings and animals with systemic inflammation (Uhlir and Whitehead, 1999; Petersen *et al.*, 2004; Harr *et al.*, 2006).

AA amyloidosis has been reported in a number of domestic and wild animal species, including dairy cows, cheetahs (*Acinonyx jubatus*), Siberian tigers (*Panthera tigris altaica*), Dorcas gazelles (*Gazella dorcas*), and rhesus monkeys (*Macaca mulatta*) (Johnson and Jamison, 1984; Blanchard *et al.*, 1986; Rideout *et al.*, 1989; Papendick *et al.*, 1997; Schulze *et al.*, 1998). In marine mammal species amyloidosis has previously been reported only in bottlenose dolphins (*Tursiops truncatus*) and Stejneger's beaked whales (*Mesoplodon stejnegeri*) (Cowan, 1995; Tajima *et al.*, 2007). The aims of the present investigation were (1) to describe the pathological features of amyloidosis in stranded California sea lions (*Zalophus californianus*), (2) to determine the chemical composition of the amyloid deposits, and (3) to identify any predisposing conditions associated with amyloid deposition.

Materials and Methods

Animals

Twenty-six cases of amyloidosis were identified by retrospective examination of formalin-fixed tissues or histological slides from 712 California sea lions (364 of which were adult animals), subjected to necropsy between 1983 and 2006. No cases were identified before 1993. One of the 26 animals with amyloidosis was included in a previous investigation on sea lion metastatic carcinoma (Gulland *et al.*, 1996b). Case material was collected from the archives of the Pathology Service, Veterinary Medical Teaching Hospital, University of California, Davis. All sea lions had been stranded live along the central California coast (37° 42'N, 123° 05'W to 35° 59'N, 121° 30'W) and transported to The Marine Mammal Center (TMMC), Sausalito, California, a rehabilitation facility. All animals were adults, as determined by standard length, tooth size, tooth dentine growth rings (Oosthuizen *et al.*, 1998), and stage of sagittal crest development. There were 24 females and two males. Clinical information was obtained from TMMC medical records.

Pathology

A post-mortem examination was completed on all animals. Selected tissue samples were fixed by immersion in 10% neutral buffered formalin. Tissues were processed by routine methods, sectioned at 4–6 µm and stained with haematoxylin and eosin (HE) or Congo red (CR). All CR-stained sections were viewed under cross-polarized light. Sections from 10 affected animals were treated with potassium permanganate 2.5% in 0.15% sulphuric acid and then with 2%

oxalic acid before staining with CR (Wright *et al.*, 1977). The amount of amyloid deposition in the kidney was graded subjectively as slight (+), moderate (++), or severe (+++).

Immunohistochemistry (IHC)

This was performed on sections (4 µm) of formalin-fixed paraffin wax-embedded kidney ($n = 3$) and liver ($n = 1$). After dewaxing, the slides were blocked with 10% normal goat serum for 30 min, drained, and incubated with a 1 in 100 dilution of the primary mouse monoclonal anti-amyloid A antibody (CM 125; Biocare Medical, Concord, CA, USA) in a moist chamber overnight at 4°C. Slides were rinsed with phosphate-buffered saline (PBS) and incubated with a biotinylated anti-mouse link reagent (Biocare Medical) for 10 min. They were then rinsed again with PBS and incubated with streptavidin horseradish peroxidase (Biocare Medical) for 10 min. Positive labelling was “visualized” with 3-amino-9-ethylcarbazole (AEC) chromogen (Zymed Laboratories, San Francisco, CA, USA). Negative controls were prepared by replacing the primary antibody with mouse myeloma IgG. A section of rhesus macaque (*M. mulatta*) spleen with amyloid A deposits was used as a positive control.

IHC for *Leptospira* spp. was performed on kidney sections of 13 animals, as above but with a *Leptospira*-specific polyclonal antibody (National Veterinary Services Laboratory, Ames, Iowa) directed against *L. interrogans* serovars bratislava, canicola, hardjo, icterohaemorrhagiae and pomona, and *L. kirschneri grippotyphosa*.

Serum Amyloid A Assay

Serum samples were obtained from seven sea lions with amyloidosis and from 35 clinically healthy yearling sea lions selected as negative controls. The samples were then dispensed in small volumes and stored at –80°C until used. The samples from animals with amyloidosis were collected at or near the time of death. Serum amyloid A was measured by a competitive ELISA with anti-bovine SAA monoclonal antibodies and with internal positive and negative controls, according to the manufacturer's recommendations (Tridelta Development Ltd, Maynooth, County Kildare, Ireland). The antibodies cross-react with SAA from most mammalian species. Basic statistics, tests of normality, graphing, and quality control statistics were performed with Minitab® 15.1.1.0, 2007 (Minitab, State College, PA, USA).

Results

Clinical Findings

Within 3 days of stranding, 21 of the 26 sea lions with amyloidosis died or were humanely killed, due to severe clinical disease and poor prognosis. Time in rehabilitation ranged from 7 to 30 days for the remaining five animals. Clinical signs in affected sea lions were generally non-specific and included severe lethargy, depression, dehydration, and anorexia. Additionally, some animals showed posterior paresis, perineal edema and abdominal pain, signs suggestive of metastatic cancer (Gulland *et al.*, 1996b). Ante-mortem blood samples obtained from 10 of the 26 animals revealed hypoalbuminaemia in them all, panhypoproteinaemia in two, and mild hyperglobulinaemia in two; eight animals had serum biochemical values indicative of renal disease, including increased concentrations of blood urea nitrogen (8/8), creatinine (6/8), and phosphorus (7/8) (Bossart *et al.*, 2001). A complete blood count (CBC) in seven of the 10 animals revealed leucocytosis and neutrophilia in five, and leucopenia with a left shift in two.

Macroscopical Lesions

Twenty-three of the 26 affected sea lions had abnormal kidneys. These were enlarged and swollen, and on cut section the cortices appeared pale tan-coloured and finely corrugated. There was loss of renicular and corticomedullary differentiation, and occasional cysts, 0.1 cm to 1.0 cm in diameter, were scattered irregularly in the cortex (Fig. 1). Thirteen animals had metastatic genital carcinoma, with lesions similar to those previously described (Gulland *et al.*, 1996b; Lipscomb *et al.*, 2000).

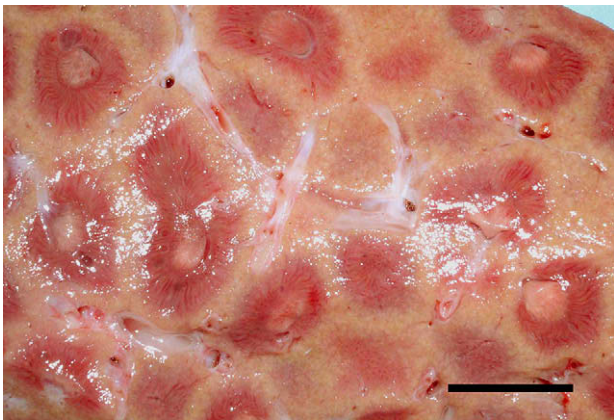


Fig. 1. Kidney from a California sea lion with amyloidosis. Note the diffusely pale and corrugated cortices, loss of corticomedullary and renicular distinction, and cysts scattered in the parenchyma. Bar, 2 cm.

Histopathology

Amyloid deposits of different degrees of severity were present in various renal sites in 24 of the 26 affected sea lions (Table 1). In 12 of these 24 animals, the kidneys showed a thick band of amyloid in the peritubular interstitium of the outer medulla, adjacent to the corticomedullary junction (Fig. 2). In HE-stained sections, amyloid appeared as pale eosinophilic, homogeneous extracellular material (Fig. 3). In CR-stained sections, amyloid appeared pale red and exhibited characteristic apple-green birefringence under cross-polarized light (Fig. 2). Pretreatment with potassium permanganate abolished CR staining, suggesting that the amyloid fibrils were of type AA (Wright *et al.*, 1977). Tubules adjacent to amyloid deposits were variably atrophied, ectatic or cystic, and were occasionally denuded of epithelium. Amyloid deposits within glomeruli consisted of segmental or nodular deposits expanding the mesangium and capillary basement membranes (Fig. 4). Glomerulosclerosis, proteinaceous tubular casts, interstitial fibrosis, and lymphoplasmacytic interstitial nephritis were concurrently present in affected kidneys.

Twenty-three of the 26 affected sea lions showed extra-renal amyloid deposition with the following distribution: small and medium sized arterioles (21 animals), thyroid gland (17), liver (3), vaginal Bartholin glands (2), spleen (1), and gastric and intestinal mucosa (3 of the 23 animals examined). The affected arterioles were located in the spleen (12 animals), brain (10), pancreas (9), adrenal gland (6), heart (6), wall of the gastrointestinal tract (6), tonsil (3), genital tract (3), and gall bladder (2), and occasionally in the trachea, tongue and pituitary gland (in each case, in one of the 23 animals examined). In the brain, arterioles in the choroid plexus and hippocampus were most commonly affected. In the thyroid gland, large deposits of amyloid were present in the interstitium separating thyroid follicles. Follicles were variably shrunken or collapsed. Hepatic

Table 1
Severity and location of renal amyloid deposits in 24 stranded California sea lions

| Severity | Number of animals with renal amyloid in | | | |
|----------|---|-----------------------|-----------|------------|
| | Medullary interstitium | Cortical interstitium | Glomeruli | Arterioles |
| + | 4/24 | 6/24 | 6/24 | 8/24 |
| ++ | 9/24 | 10/24 | 11/24 | 15/24 |
| +++ | 9/24 | 7/24 | 4/24 | 0/24 |
| Totals | 22/24 | 23/24 | 21/24 | 23/24 |

+, Slight; ++, moderate; +++ abundant.

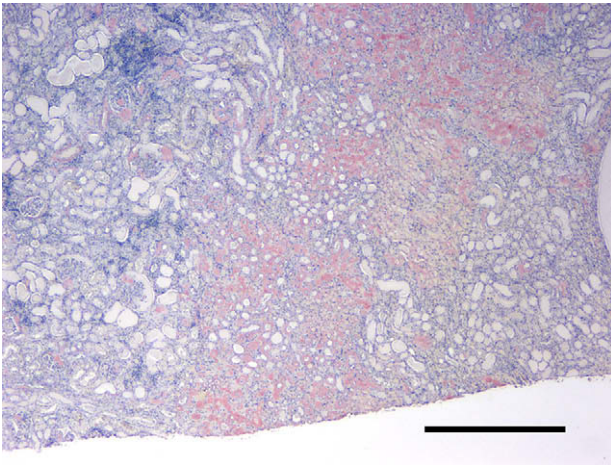


Fig. 2. Marked amyloid deposition in the outer medulla of the kidney adjacent to the corticomedullary junction. Congo red. Bar, 1 mm.

amyloidosis was characterized by deposits within the space of Disse, primarily in the mid-zonal region. In severely affected areas, hepatic cords were compressed and atrophied. In three cases, mural gastric vascular amyloid deposits were located in areas of parasite-associated ulcers. In one sea lion, amyloid deposition was restricted to blood vessels adjacent to a region of severe balanoposthitis.

Immunohistochemistry

Immunolabelling was performed on three cases of renal amyloidosis and one case of hepatic amyloidosis. In all cases, amyloid deposits exhibited moderate red-brown labelling with antibodies against amyloid A (Fig. 5).

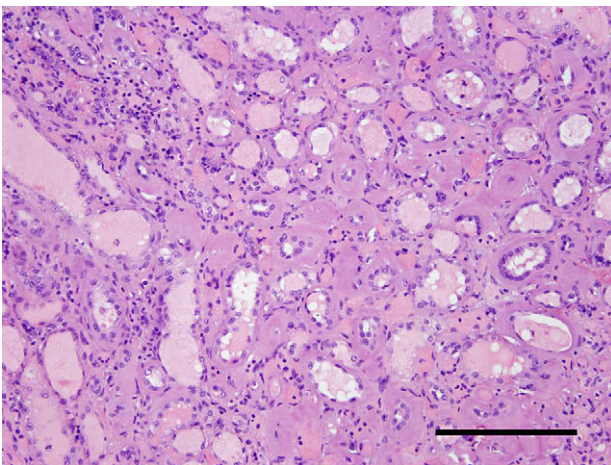


Fig. 3. Peritubular amyloid deposition within the renal interstitium at the corticomedullary junction. HE. Bar, 200 μ m.

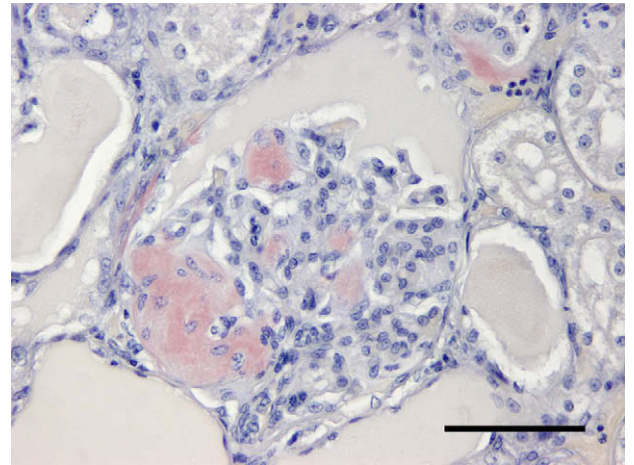


Fig. 4. Nodular amyloid deposits expanding the mesangium of a glomerulus. Congo red. Bar, 100 μ m.

Associated Diseases

Chronic inflammatory diseases, often multiple, were common in the sea lions with amyloidosis (Table 2), as they are in most stranded sea lions. Thirteen (50%) of the 26 sea lions had concurrent metastatic carcinoma, typical of endemic metastatic carcinoma in California sea lions (Gulland *et al.*, 1996b), recently determined to be of genital origin (Lipscomb *et al.*, 2000). All animals with genital carcinoma were female. Tumours originated in the vagina or cervix and metastases were present in various sites that included regional lymph nodes, and abdominal and thoracic viscera. Intense lymphocytic inflammation was present in affected genital epithelium.

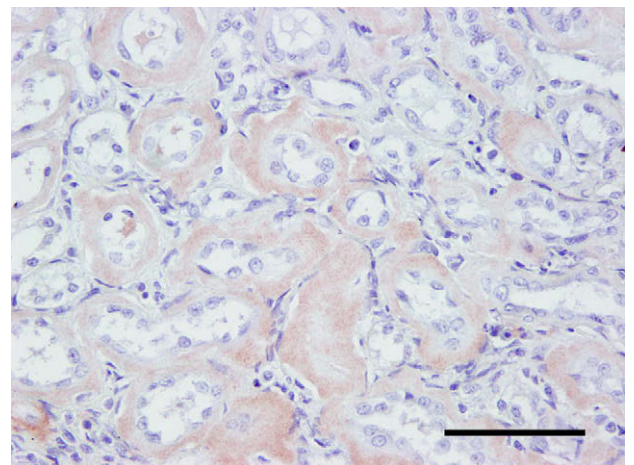


Fig. 5. Positive immunohistochemical labelling of amyloid deposits in the renal interstitium at the corticomedullary junction with an amyloid A monoclonal antibody. Streptavidin–biotin–peroxidase complex. IHC. Mayer's haematoxylin counterstain. Bar, 100 μ m.

Table 2
Concurrent inflammatory diseases in 26 California sea lions with amyloidosis

| Concurrent inflammatory disease | Number of animals |
|---------------------------------|-------------------|
| Genital carcinoma | 13 |
| Cholecystitis | 7 |
| Gastritis/Gastric ulceration | 8 |
| Enterocolitis | 4 |
| Verminous pneumonia | 4 |
| Chronic abscesses | 2 |
| Balanoposthitis | 1 |
| Leptospirosis | 1 |
| Osteomyelitis | 1 |
| Trematode-associated hepatitis | 1 |

Tumour metastases often contained areas of central necrosis and widespread inflammation. Multifocal lymphoplasmacytic inflammation was present in some affected kidneys; however, immunolabelling demonstrated leptospirosis in only one of 13 sea lions with amyloidosis and concurrent lymphoplasmacytic interstitial nephritis. Mild to moderate adrenal cortical hyperplasia was present in 13 (50%) of 26 affected sea lions.

Serum Amyloid A Assay

Serum amyloid A data were not normally distributed ($P < 0.01$), according to a Kolmogorov–Smirnov test of normality. Sea lions grouped (before SAA assay) as amyloidosis-affected or apparently healthy animals (not showing signs of amyloidosis) had median SAA concentrations of $>1200 \mu\text{g/ml}$ (range 28 to >1200) and $<10 \mu\text{g/ml}$ (range <10 to >1200), respectively (Fig. 6). The values for diagnostic sensitivity and specificity, based on the same groups of animals, were 78% and 86%, respectively. Of the seven amyloidosis-affected sea lions subjected to SAA assay, six

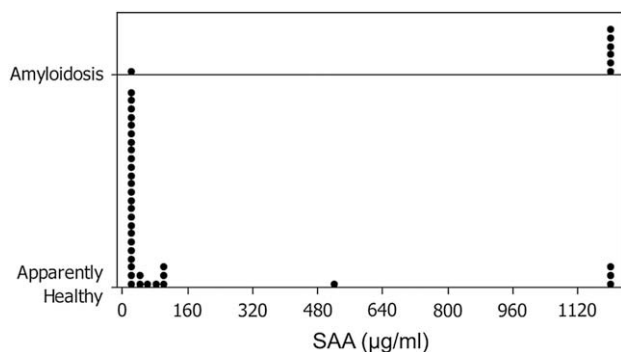


Fig. 6. SAA concentration ($\mu\text{g/ml}$) in a subset of seven sea lions in which amyloidosis was diagnosed at necropsy (top) and 35 negative control sea lions assessed as clinically healthy with no signs of amyloidosis (bottom).

had SAA concentrations of $>1200 \mu\text{g/ml}$. The remaining sea lion had an SAA concentration of $28 \mu\text{g/ml}$; in this animal amyloid deposition was localized to blood vessels in regions of balanoposthitis, which appeared to be the only inflammatory disease present. Retrospective examination of case histories revealed that all apparently healthy animals showing increased SAA concentrations had either deep wounds with abscesses (4/9), signs of resolving pneumonia (3/9), or corneal ulceration (2/9).

Discussion

In this study, 7% of the adult live stranded sea lions had amyloid deposits in one or more tissues. Although stranded marine mammals represent a skewed sample of the free-living population and the prevalence of diseases is not the same as in the entire population, this study suggests that amyloidosis may contribute to morbidity and mortality in adult California sea lions. In view of the frequent involvement of the kidneys, amyloidosis should be considered in the differential diagnosis of renal disease in adult sea lions, especially in animals with concurrent hypoalbuminaemia.

Immunohistochemical and histochemical techniques revealed that the amyloid deposits were formed from SAA-derived protein and could be classified as AA amyloid. Reactive systemic AA amyloidosis is usually secondary to systemic inflammation or neoplasia, or both, and most of the affected sea lions had concurrent inflammatory diseases. The most frequent of these was metastatic genital carcinoma, which is a common cause of mortality in older stranded California sea lion, affecting approximately 18% of such animals. There is often severe inflammation in the tissue adjacent to and within tumours, and secondary bacterial infection is common (Gulland *et al.*, 1996b; Lipscomb *et al.*, 2000). Sea lion genital carcinomas have been associated with a gammaherpesvirus (otarine herpesvirus-1 [OthV-1]), and intranuclear viral inclusions can occasionally be found in neoplastic epithelium (Lipscomb *et al.*, 2000; King *et al.*, 2002). Tumour-associated amyloid deposition has been reported in squamous cell carcinomas of the human cervix and vagina, which are associated with human papilloma virus (HPV) infection (Hsueh and Kuo, 1986; Tsang and Chan, 1993). Localized amyloid deposition has also been noted in human nasopharyngeal carcinomas, which are associated with a gammaherpesvirus, Epstein Barr-virus (Prathap *et al.*, 1984). SAA concentrations are sometimes increased in patients with this disease (Cho, 2007). Only one of the female sea lions with concurrent genital carcinoma had amyloid deposition in the genital

tract. These deposits were located in small submucosal arterioles adjacent to affected epithelium; amyloid was never located within the tumour interstitium, in contrast to amyloid-producing genital carcinomas in women and nasopharyngeal carcinomas. The secondary inflammatory lesions in sea lions with genital carcinoma may have accounted for the amyloid deposition. The potential relationship between herpesvirus infection and amyloidosis is unclear. Leptospirosis is a common cause of interstitial nephritis in free-ranging sea lions and these animals are suspected to be chronic shedders of the bacterium (Gulland *et al.*, 1996a); however, renal interstitial inflammation was not usually due to leptospiral infection. Many of the other concurrent inflammatory diseases identified, such as cholecystitis, gastritis and enterocolitis, are common findings in wild sea lions and are usually associated with parasitism (Daily, 2001). Such inflammatory conditions, although usually considered clinically insignificant in stranded California sea lions, might theoretically cause release of amyloidogenic cytokines.

In blood, SAA has a short half-life (<2 days) and concentrations are therefore increased only during periods of active, systemic inflammation (Lozanski *et al.*, 1996). It has been used as a highly sensitive and specific diagnostic indicator of active generalized inflammation in numerous species, such as dogs, horses, cows and manatees (*Trichechus manatus latirostris*) (Hulten and Demmers, 2002; Petersen *et al.*, 2004; Harr *et al.*, 2006; Kjølgaard-Hansen *et al.*, 2007). SAA concentrations were high in most of the amyloidosis-affected sea lions tested, confirming the presence of inflammation. SAA was shown to be increased in other wild species with confirmed AA amyloidosis, including pig-tailed macaques (*Macaca nemestrina*) and Siberian tigers, as well as domestic species such as the cow and the horse (Schulze *et al.*, 1998; Hukkanen *et al.*, 2006; Takahashi *et al.*, 2007). Localized lesions, such as the balanoposthitis noted here or flipper lesions in manatees, do not typically result in increased SAA concentration (Harr *et al.*, 2006).

Approximately 70% (24/35) of the animals assessed as clinically healthy, with no signs of amyloidosis, showed no more than trace concentrations of SAA, typical of unstressed healthy animals. Nine animals, however, had SAA concentrations that exceeded the established reference value (50 µg/ml). Although clinically healthy at the time of examination and blood collection, incompletely resolved inflammation may have been present in these animals. Three apparently healthy animals not exhibiting signs of amyloidosis, with SAA concentrations of >1200 µg/ml, had a history of deep traumatic wounds and tissue abscesses. Assessment of complete resolution of this type of lesion

is also difficult in some other species, such as the manatee. Two animals with mid-range concentrations (between 100 and 800 µg/ml) had pneumonia, a disease well known to stimulate the acute phase response (Ceron *et al.*, 2005). All nine animals had normal concurrent CBCs, suggesting that SAA is a more sensitive indicator of inflammation in sea lions, as it is in other species (Ceron *et al.*, 2005; Lycopoulou *et al.*, 2005). However, further analysis of SAA in sea lions with different inflammatory conditions is needed to determine its clinical usefulness.

Other factors that may predispose animal species or individual animals to reactive systemic amyloidosis include (1) variation in the production or enzymatic degradation of SAA protein, and (2) structural SAA polymorphisms that may be particularly amyloidogenic (DiBartola and Benson, 1989). In Abyssinian cats, predisposition to amyloidosis is thought to be related to a retained intact conserved region within the apoSAA precursor protein (Kluve-Beckerman *et al.*, 1989). It is unknown whether any of these factors play a role in development of amyloidosis in California sea lions. Stress has been proposed as a factor in development of amyloidosis in other species (Cowan and Johnson, 1970; Germann *et al.*, 1990; Papendick *et al.*, 1997). In this study, however, only 50 % of the affected sea lions had concurrent adrenocortical hyperplasia, in contrast to its much higher prevalence in cheetahs with systemic amyloidosis. In cheetahs, stress has been suggested as a factor in amyloid deposition (Papendick *et al.*, 1997).

The tissue tropism of amyloid deposition in affected sea lions was similar to that reported in reactive systemic amyloidosis in other species (DiBartola and Benson, 1989). The tendency for amyloid to be deposited in a band along the outer medulla was a striking finding. Renal amyloid deposits in bottlenose dolphins were also reported to occur predominantly in the corticomedullary region (Cowan, 1995). The basic renal architecture is similar in cetaceans and pinnipeds, the kidneys being subdivided into numerous reniculi separated by thin connective tissue septa. Each reniculus contains a cortex, medulla, medullary pyramid and calyx. In cetaceans, there is a discontinuous band of connective tissue and smooth muscle at the corticomedullary junction, termed the intrarenicular sporta. This band is absent in pinniped kidneys. In both groups, however, numerous vasa recta occur throughout the outer medulla and corticomedullary junction. Dense amyloid deposits surrounding the vasa recta of the outer medulla have been reported in some cases of human renal amyloidosis (Nakamoto *et al.*, 1984). The underlying reason for the different distribution of amyloid deposits in different species is poorly understood. Proteoglycans are an important

component of all amyloid deposits and play a role in the genesis and stabilization of amyloid fibrils. These proteoglycans, which have a certain degree of structural and biochemical heterogeneity depending on the tissue, may play an important role in tissue tropism (Bellotti *et al.*, 2007). Differences in chemical structure may also play a role. In human patients, Westermark *et al.* (1979) demonstrated differences in the chemical composition of protein AA between medullary and glomerular amyloid deposits.

In conclusion, this study demonstrated systemic amyloidosis in California sea lions, primarily affecting the kidneys and often related to concurrent inflammatory or neoplastic diseases. Further studies on the factors that play a role in amyloid formation in sea lions are needed. In particular, amyloid protein purification and sequence analysis would assist in determining the SAA isoforms that occur in sea lion amyloidosis.

Acknowledgments

We thank the staff and volunteers of The Marine Mammal Center for care of the animals, especially Liz Wheeler for help with sample collection and photography. The UCD VMTH pathology residents are acknowledged for initial histological examination of some of the cases. Ms Melanie Pate is acknowledged for technical support and SAA data production. Financial support for F.M.D. Gulland was provided by the Arthur and Elena Court Nature Conservancy. K.M. Colegrove was supported by grant ESO70555 from the National Institute of Environmental Health Sciences (NIEHS).

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[Received, September 20th, 2007]
 [Accepted, August 27th, 2008]