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A new model of AA-amyloidosis induced by oral pristane in BALB/c mice

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Summary. Fifteen male BALB/c mice were given six intermittent oral doses of 0.1 ml pristane (2, 6, 10, 14 tetramethylpentadecane) within a period of 9 weeks. Fifteen mice receiving tap water using the same schedule formed the control group. Amyloidosis was first detected in the spleen of a mouse which had died 33 weeks after the first dose and 24 weeks after the last. All six mice which were subsequently autopsied 34–51 weeks after the first dose also showed amyloidosis involving liver and spleen. The most extensive tissue deposits were seen at 37–38 weeks whereas the older mice showed predominantly chronic renal lesions with papillary necrosis, scars and cystic change. Electron microscopy confirmed the identity of the amyloid fibrils and the presence of globular stellate amyloid 'bodies' in liver and spleen. The amyloid deposits were shown to be made up of AA (amyloid associated) protein using an indirect immunoperoxidase method and a monoclonal rat anti-murine AA protein antibody. We did not find any plasmacytomas or increased numbers of plasma cells in the bone marrow. None of the control mice developed amyloidosis. This new experimental model promises to provide a means of studying several aspects of secondary amyloidosis which may be relevant to the clinical situation.

Keywords: secondary amyloidosis, amyloid associated (AA) protein, BALB/c mice, globular amyloid, pristane

In a study designed originally to produce a model of primary gastrointestinal lymphomas, we fed BALB/c mice with intermittent doses of pristane (2, 6, 10, 14 tetramethylpentadecane), a chemical known to produce plasmacytomas on intraperitoneal injection into this strain of mice (Potter & Wax 1983). Although we did not produce any lymphomas or plasmacytomas, all the mice which were killed or died at 33 weeks or more after the first dose, developed amyloidosis instead. We now report the findings in this new experimental amyloidosis model, and highlight some of its interesting features.

Materials and methods

Induction method. Male BALB/c mice reared in the Laboratory Animal Unit of the University of Hong Kong were used. Fifteen mice aged 4–6 weeks were given 0.1 ml pristane (Sigma Chemical Co. Ltd.) via a syringe and needle, with a round bulb at the tip, introduced into the lower oesophagus. The

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second dose was given after an interval of 1 week and thereafter, four further doses were given at 2 week intervals. Fifteen control mice of the same age and sex received tap water using the same procedures. The two groups of mice were housed in wire cages and given water and 'Purina' laboratory chow *ad libitum.*

Autopsy and histological procedures. Animals (test and controls) were killed at intervals by cervical dislocation and autopsy performed. Liver, spleen, mesenteric lymph node and portions of stomach and intestines were fixed in buffered formalin for preparation of paraffin sections in all cases, with a block of the unfixed spleen frozen in liquid N_2 and kept at $-70°C$. Where there was grossly detectable abnormality in the other organs, these were sampled as well. After examination of the haematoxylin and eosin stained sections revealed changes highly suggestive of amyloidosis, Congo Red staining was done on the liver and spleen sections of all the mice, on all available kidney specimens, and on mesenteric lymph node sections as indicated. Grading of the extent of amyloid deposition in the spleen was done as described by Scheinberg *et al.* (1976*a*). The liver deposits were graded as follows:

±, occasional deposits found after careful search;
+, scattered sinusoidal deposits in less than half the liver lobules;
++, small deposits in most of the liver lobules;
+++, extensive deposits in every lobule, with atrophy of the hepatocytes.

Because amyloidosis was an unexpected finding, sampling of organs such as the kidney and heart for microscopical examination did not form part of the original autopsy protocol.

Electron microscopy. After confirmation of amyloidosis by microscopic examination, small blocks of liver and spleen from subsequent autopsies were fixed in glutaraldehyde and processed for electron microscopy by dehydration in a graded series of ethanol, embedded in Epon and ultrathin sections were stained with uranyl acetate and lead citrate.

Immunohistochemistry. Immunohistochemical identification of the amyloid protein was carried out on 5 μm cryostat sections of spleen from three amyloidotic animals using an indirect immunoperoxidase technique (Livni *et al.* 1980). The dried sections were fixed in acetone for 5 min. A rat (IgM) monoclonal antibody against murine AA (amyloid associated) protein (kindly provided by Dr M. Benson of the Indiana University School of Medicine) was used as the primary antibody, followed by a peroxidase conjugated swine anti-rat IgM from Serotec Labs., Oxford. The peroxidase reaction was developed using diaminobenzidine and H_2O_2 and sections counterstained with haematoxylin. Control sections omitting the primary antibody were used, and spleens from two mice in the control group were also examined with the same method.

Results

Table 1 shows the time of appearance of amyloid deposits in the spleen, liver and other organs. Mice which had been killed at intervals during the period of induction (weeks 1–8) showed no evidence of amyloidosis or other significant pathology. The earliest detectable deposits were found at the periphery of the white pulp in the spleen of Mouse no. 9, which had died 33 weeks after the first dose of pristane. The eosinophilic deposits were confirmed to be amyloid by demonstrating green birefringence after Congo Red Staining. Thereafter, all the mice showed evidence of amyloidosis, with the most extensive deposits seen in Mouse nos 11 and 12. In the liver, the deposits were found in the sinusoids as well as the walls of arteries and veins (Fig. 1). Mouse no. 11 was the only one which showed extensive deposits of amyloid around the medullary sinuses of the

Table 1. Incidence of amyloid at various sites after oral administration of pristane

			Major sites of amyloid deposition*				
					Kidney		
Mouse no.	Age (weeks)	Interval after 1st dose (weeks)	Spleen	Liver	Amyloid	Other changes	MLN
1–6	5–14	1–8	–	–	NE/–	N	NE/–
7	23–25	19	–	–	–	N	–
8	30–32	26	–	–	NE	N	–
9†	37–39	33	++	±	NE	N	NE
10	38–40	34	++	+	NE	N	+
11	41–43	37	+++	++	++	N	++
12	42–44	38	++++	+++	NE	N	NE
13†	44–46	40	++	+	+	Scarred, PN	±
14†	55–59	51	+++	±	+	Cystic, PN	–
15	55–59	51	+	±	±	Small, cystic scarred	±

* Confirmed by Congo Red Staining.
MLN Mesenteric lymph node.
NE Not examined histologically, or in MLN, inadequate tissue sample.
N Grossly normal.
PN Papillary necrosis.
(–) Negative; (±) to (++++) Grades of amyloid deposition (see text).
† These mice had died.

mesenteric lymph node and in the interstitium of the kidneys, with smaller amounts in the glomeruli. We observed with interest a diminution in amount of amyloid in the organs of the last three mice (nos 13–15) compared to Mouse nos 11 and 12, particularly in view of the fact that severe renal changes in the form of necrosis of the renal papillae, scarring and cystic changes were found in all these older mice (Fig. 2). These renal changes are similar to those previously described in amyloidosis occurring as a primary disorder in several inbred strains of mice (Dunn 1967).

The pattern of splenic amyloid deposition was similar to that described in many experimental models (Cohen *et al.* 1959; Heefner & Sorenson 1962; Werdelin & Ranløv 1966; Scheinberg *et al.* 1976*b*). The amyloid formed a partial or complete rim at the periphery of the splenic white pulp, sometimes wrongly referred to as a 'perifollicular' distribution. Small rounded 'stellate' deposits with radially distributed fibres were also seen, usually at the edges of the ring-like deposits. In the severely involved spleens, widely confluent areas of amyloid replaced most of the splenic tissue. Figure 3 shows the result of immunoperoxidase staining of a splenic section of Mouse no. 13 with the rat anti-mouse AA protein antibody. The rim of positively stained material around the splenic white pulp is identified as AA protein. The same method also stained the globular stellate deposits, but confirmed the absence of AA protein in the control mice.

Electron microscopy of the liver and splenic deposits showed abundant randomly orientated intercellular non-branching fibrils of approximately 10 nm width, typical of amyloid. In addition, there were more well organized globular deposits, with the fibrils orientated in a radial fashion towards a central core (Fig. 4). These were found in

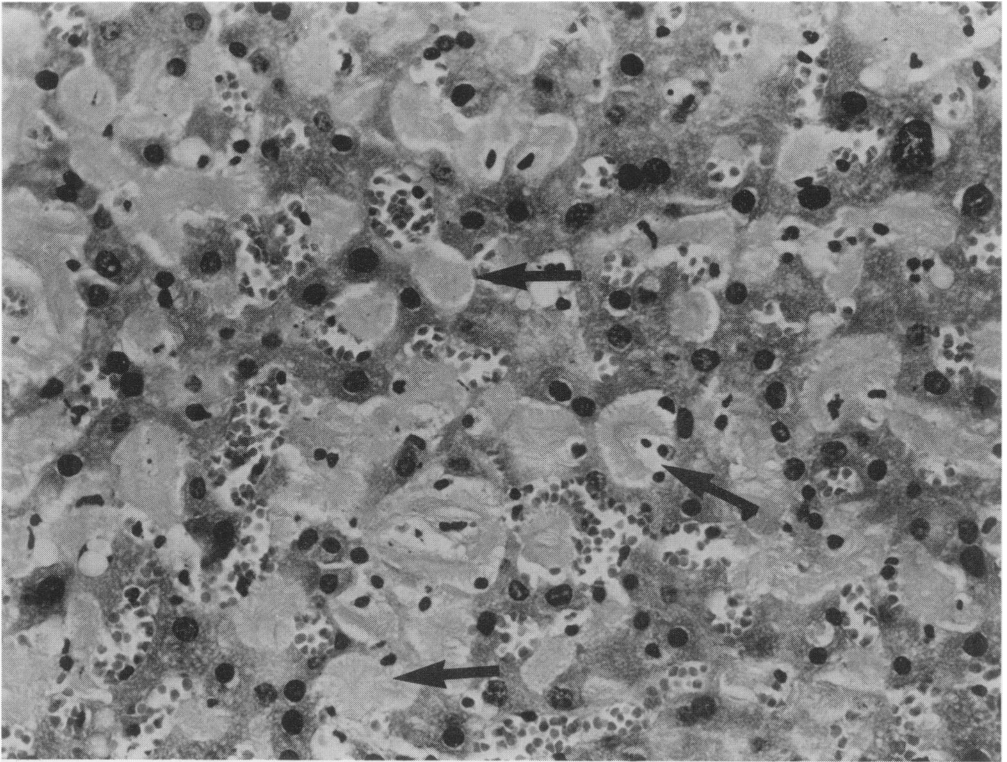

Fig. 1. Liver of Mouse no. 12. The sinusoidal spaces are expanded and the liver cells appear atrophic due to deposition of amyloid. In many areas, the amyloid fibrils are orientated in a distinctive manner, radially, pointing towards a central core (arrows). H & E, ×380.

both liver and spleen and are similar to those stellate fibrillary structures described in casein induced amyloidosis (Heefner & Sorenson 1962; Sorenson *et al.* 1964; Janigan & Druet 1966), including splenic cell cultures (Bari *et al.* 1969). We did not observe an obvious relationship of those deposits to any particular cell. In the liver, these 'globules' were seen within the space of Disse.

These were no significant deposits in the gastrointestinal tract, nor in the hearts of two mice which were examined histologically. There was no evidence of plasmacytomas, myelomas, or lymphomas in any of the mice and plasma cell numbers were not increased in the bone marrow. None of the control group of mice developed amyloidosis.

Discussion

Although the pattern of amyloid deposition in the tissues closely resembled previous experimental models which have been shown to be examples of secondary, or AA amyloidosis (Skinner *et al.* 1977), it was particularly important, in the present model, to confirm the nature of the amyloid protein. This is because pristane is known to be a substance which induces plasmacytomas, and therefore there was a possibility that it could produce amyloidosis of immunoglobulin light chain origin (AL amyloid), either associated with plasmacytoma or myeloma, or in a 'primary' form. However, the immunohistochemical identification of the amy-

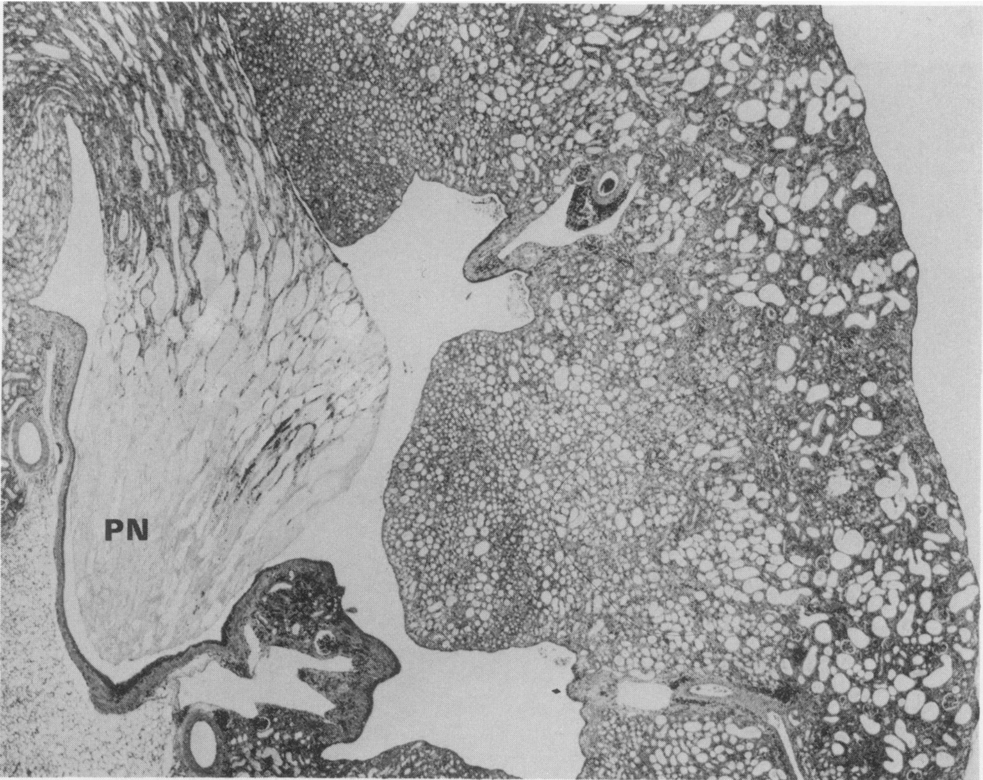

Fig. 2. Kidney of Mouse no. 13 showing papillary necrosis (PN). There is coarse scarring and cystic dilatation of the tubules. H & E, × 25.

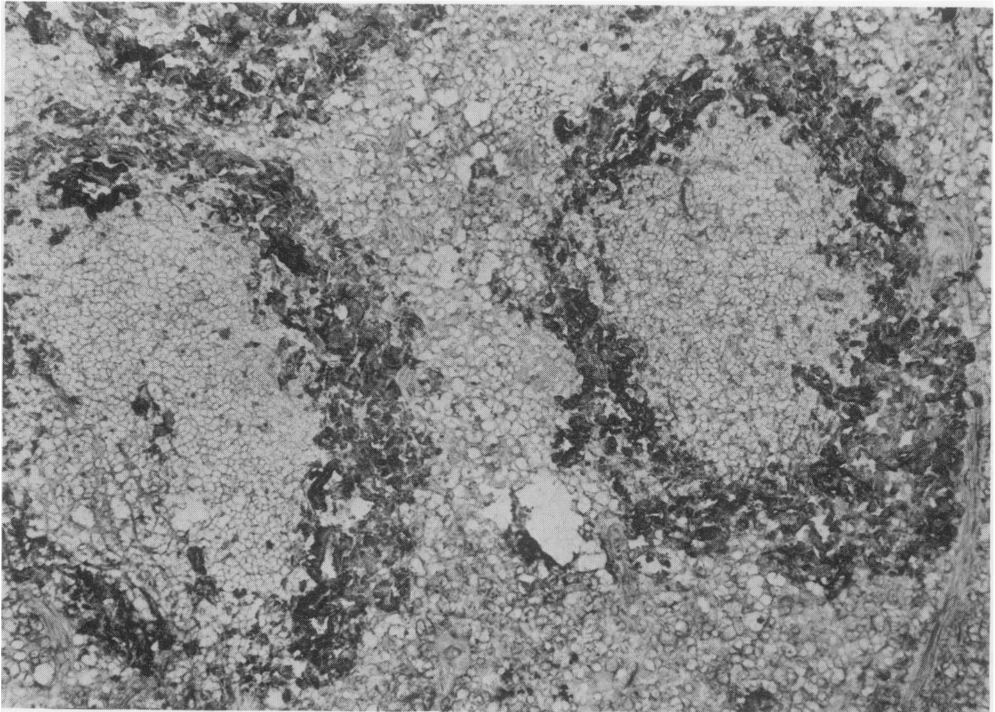

Fig. 3. Immunoperoxidase staining of frozen section of spleen for murine AA protein. The amyloid deposits forming a rim around the white pulp are stained positively. Mouse no. 13, × 160.

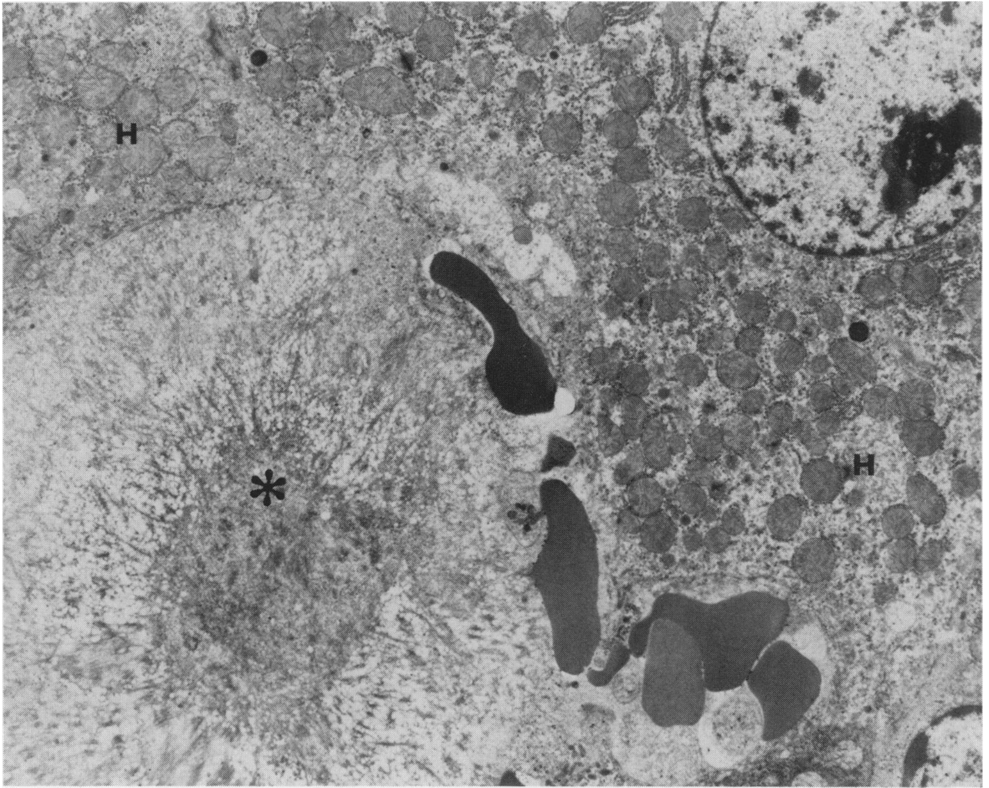

Fig. 4. Electron-micrograph of liver from Mouse no. 13 showing a 'globular' deposit of amyloid(*) within the space of Disse. The fibrils are orientated radially. Red blood cells are seen inside a compressed sinusoid, between the deposit and several hepatocytes (H). ×4700.

loid to be of AA type, together with lack of increased numbers of plasma cells in the marrow, or plasmacytomas elsewhere, is taken as evidence against an AL amyloidosis in this experimental model.

The mechanisms whereby pristane produces an AA type amyloid is unknown. One observation made during the course of these experiments which may be relevant in this respect was that all the mice receiving oral pristane showed a reaction starting from about 30 min after administration. The mice became agitated and their fur moist. Instead of their usual habit of huddling together, each ran to a separate corner of the cage. After a day or so, the mice returned to normal and appeared to suffer little sustained effects. This reaction may be significant since AA protein is believed to be derived by proteolytic cleavage from a serum precursor, the SAA protein, which is an acute phase reactant (McAdam & Sipe 1976; Benson *et al.* 1977). It is also worth noting that BALB/c mice receiving an intraperitoneal injection of 0.5 ml pristane were never (in our experience) observed to have a similar reaction and amyloid has not been detected in such mice. The repeated administration (via the oral-gastric route) of pristane may have been responsible for stimulating a series of reactions wherein acute phase proteins were released in large amounts into the circulation. We are now repeating these experiments and taking blood samples specifically to look for evidence of this.

Although many experimental models of

secondary amyloidosis have been described and well studied, this particular model shows several interesting features which may be worthy of further study. Firstly, the amyloidosis develops slowly, over a period of time, which, compared to the human situation, would be the equivalent of a period of several years. Many of the established experimental models depend on repeated parenteral injections e.g. of casein, with amyloidosis appearing during the time when injections are given, often within a period of 4 days to 4 weeks in mice (Cohen & Shirahama 1972; Suzuki *et al.* 1980). This may therefore be a more appropriate model to study the mechanisms of production of secondary amyloidosis, which in the human situation, also takes a period of years to develop. Although pristane is known to occur in nature, particularly in marine organisms (Avigan *et al.* 1967), and therefore could be ingested in small amounts by humans via edible seafood and mineral oils, the very small amounts ingested (e.g., shark liver was found to contain 2.0–2.4 μg/g of pristane (Avigan *et al.* 1967), whereas 0.1 ml of pristane from Sigma contains 78.5 mg) is unlikely to produce significant reactions even if these do occur in humans. However, it is possible that repeated episodes of inflammatory reactions, initiated by infective or chemical agents may have comparable modes of action as pristane in inducing secondary amyloidosis, and even at an interval after the last exposure to the stimulating agent.

Secondly, there is a suggestion, from observing the extent of tissue deposition in the mice over a period of time, that reabsorption of the amyloid may have occurred in the model under study. The fact that severe renal damage occurred in all the three older mice despite insignificant amyloid deposits in at least two of the three mice may mean, either that renal damage may result from only small amounts of amyloid or, alternatively, that at some earlier stage those three older mice had accumulated much more significant tissue deposits, but that these had been partially reabsorbed. The small number of animals involved in these preliminary studies does not allow us to draw firm conclusions. However, reabsorption of experimentally induced amyloidosis has definitely been demonstrated in the rabbit (Richter 1953) and, if our findings can be confirmed, this murine model may be a useful one to study the mechanisms of reabsorption of amyloid which would have obvious relevance to the clinical situation.

Lastly, this model appears to conveniently combine within one animal a range of morphological features, at light and electron-microscopic level, which have previously been described in a number of separate experimental situations. This includes the characteristic globular/stellate deposits in both liver and spleen which are recognizable by light microscopy, but much more striking on electron microscopy, and which for some unknown reason are common in the experimental situation, regardless of the mode of induction, but not in clinical disease. The fact that similar 'globular' deposits have been described in human liver, albeit rarely (French *et al.* 1981) would indicate that they are not irrelevant experimental 'artefacts'. The mechanisms producing a relative preponderance of interstitial, rather than glomerular lesions in the kidney would also be worth studying. This model promises to be a useful one to examine both the individual factors as well as the interrelationships between the many factors which, together, produce the multifaceted features of secondary amyloidois.

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