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Diagnosis of the type of amyloid in paraffin wax embedded tissue sections using antisera against human and animal amyloid proteins *

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Summary. Different histochemical techniques were compared on paraffin wax embedded tissue sections for routine classification of amyloid; the following methods were used: potassium permanganate, the indirect immunoperoxidase method using polyclonal anti-human amyloid antisera (anti-AA, anti-Aλ, anti-Aκ and anti-AF) and the peroxidase-antiperoxidase (PAP) method using antisera against human, bovine, hamster and canine AA amyloid. Anti-human AA antiserum appeared to be a useful tool in this respect. Polyclonal anti-AL antisera may be helpful in diagnosing AL amyloid, but were of less value than anti-AA serum.

Strong cross reactivity between anti-bovine AA antiserum and human AA amyloid deposits was found. This indicates that animal amyloid AA antisera can also be used for the diagnosis of AA amyloid in human tissues.

Key words: Amyloid – Potassium permanganate – Immunoperoxidase

Introduction

Amyloidosis is a disease complex characterized by extracellular deposits of amyloid fibrils. These fibrils show chemical diversity depending on the underlying disease. One common feature, the β-pleated sheet conformation (Glenner and Page 1976), accounts for the characteristics of amyloid: congophilia and green birefringence, resistance to proteolysis and insolubility in physiological solutions. Different clinicopathological entities exist and a variety of investigations has led to classifications based upon the clinical pat-

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tern of organ dysfunction (Van Rijswijk and Van Heusden 1979), on the chemical nature of the protein fibrils and their precursors (Glenner et al. 1980), and on the clinicopathological conditions related to chemical types (Glenner 1980). Table 1 proposes a summary of these classifications.

The clinically important forms of systemic amyloid consist of AA proteins, AL proteins or AF proteins. AL-amyloid is derived from immunoglobulin light chains, including either lambda types (Aλ) or kappa types (Aκ). It is associated with immunocyte dyscrasia, classically with myeloma (Kahler's disease). It also occurs in an idiopathic form, although in many such cases an underlying plasma cell dyscrasia can also be demonstrated (Osserman 1961a, b). Since AL-amyloid consists mainly of the N-terminal variable region of a homologous light chain, which differs in each patient, there is little immunological cross reactivity between patients for immunohistology and thus a battery of antisera is needed (Glenner et al. 1970; Cornwell et al. 1977; Linke et al. 1981; Linke and Nathrath 1982; Linke et al. 1984a). If, in a number of cases, cross reactivity is found, this might be accounted for by remnants of constant regions attached to the variable light chain regions (Westermark et al. 1981).

AA-amyloid, occurring in reactive (secondary) systemic amyloidosis, is derived from a plasma precursor protein, called SAA. In contrast to AL-amyloid these AA protein fibrils are found to be almost identical between patients (Benditt and Eriksen 1977). Therefore, using an anti-AA antiserum, immunohistochemical demonstration of AA-amyloid in tissue sections is possible (Gruys and Timmermans 1979; Fujihara et al. 1980; Linke et al. 1981; Shirahama et al. 1981; Shirahama et al. 1981; Hind et al. 1983; Orfila et al. 1983; Linke 1984). Furthermore, the AA-amyloids found in animals resemble that found in man. Extensive chemical homologies of protein AA among different species have been described (Eriksen et al. 1976; Gorevic et al. 1977; Waalen et al. 1980; Gorevic et al. 1982), as well as immunohistochemical cross reactivity, although in the latter a variability in reaction intensity was found (Doepel et al. 1981; Linke et al. 1984b; Linke et al. 1985).

It is suggested that not all amyloid precursor proteins are equally amyloidogenic, specific subclasses being more likely to be deposited as amyloid than others. In AL-amyloidosis a predominance of λ light chains in both the amyloid deposits and in the sera and urine of these patients exists in contrast to k chain predominance in myeloma proteins of non-amyloidotic myeloma cases as well as in normal immunoglobulins (Husby et al. 1974; Isobe and Osserman 1974; Franklin 1980; Franklin and Gorevic 1980; Isobe et al. 1983; Orfila et al. 1983). There is one rare subclass of λ chains (Vλ VI) which appears almost exclusively in association with amyloidosis (Solomon et al. 1982).

In prealbumin-related amyloid investigations to date have shown that only a single amino acid was altered in the amyloidogenic prealbumin of the patients with familial neuropathic amyloidosis (Saraiva et al. 1983; Tawara et al. 1983; Dwulet and Benson 1984). Recently microheterogeneity in both SAA and AA proteins has been described. Variations in size, charge

Table 1. Classification of Major Amyloid Disseases

Type of amyloid	Characteristic protein	Precursor protein	Associated clinicopathologic process	Clinical pattern of organ dysfunction
AA	Protein AA	SAA	Reactive Systemic Amyloidosis	
			recurrent inflammation (e.g. rheumatoid arthritis, t.b.c.)	'Typical' distribution: nephropathy
			malignancies (e.g. renal cell carcinoma, Hodgkin's disease)	
			Familial Mediterranean Fever	
			Idiopathic Systemic Amyloidosis (with 'typical' distribution pattern)	
AL	$V_{\lambda,\kappa}$	Ig light chain	Immunocyte Dyscrasias	'Atypical' distribution: nephropathy cardiomyopathy glossopathy neuropathy arthropathy myopathy
			e.g. multiple myeloma	
			e.g. macroglobulinaemia	
			Idiopathic Systemic Amyloidosis (with 'atypical' distribution pattern)	
			Extramedullary Solitary Plasmocytoma ('amyloid tumor')	Localized
AF_p	Prealbumin fragments	Prealbumin	Familial Systemic Neuropathic Amyloidosis	'Atypical'
			e.g. Portugese	
AS_{c_1}, AS_s	Prealbumin fragments	Prealbumin	Senile Cardiac and Systemic Amyloidosis	'Atypical'
AS_{c_2}	Unique protein	–	Senile Isolated Atrial Amyloid	Localized
$AS_{b_1 b_2}$	–	γ trace prot./ neurofilaments	(Pre)senile Brain Amyloid (vascular/plaques)	Localized
AE	Hormone polypeptide	(pro)hormone	APUD-Endocrine Tissue Related Amyloid	Localized
			e.g. medullary c.a. of thyroid,	
			e.g. pancreatic islets in D.M.	
AD	–	Keratin?	Cutaneous Amyloid	Localized

AA Amyloid, type A
AL Amyloid, light chain derived
AF_p Amyloid, Familial Portugese type
$AS_{c,s,b}$ Amyloid, Senile type; cardiac, systemic, brain
AE Amyloid, Endocrine type
AD Amyloid, Dermal type
SAA Serum precursor protein of amyloid A fibrils
$V_{\lambda,\kappa}$ Variable region of λ or κ light chains

(Linke et al. 1975; Gorevic et al. 1978; Bausserman et al. 1980; Bausserman et al. 1982; Hol et al. 1984; Marhaug et al. 1984) and even in amino acid sequence (Eriksen and Benditt 1980; Hoffman and Benditt 1982; Hoffman et al. 1984; Eriksen and Benditt 1984) have been found which might be related to some SAA types being more amyloidogenic than others (Gorevic et al. 1978). In renal amyloidosis without glomerular involvement a protein AA of larger size appears to be deposited than that deposited in cases with glomerular involvement (Westermark et al. 1979).

Classification of amyloid is important because of different therapeutic regimens, which may suppress the underlying disease and thus amyloidosis. The potassium permanganate method is routinely used to differentiate AA-amyloid from non-AA-amyloid. After pretreatment with potassium permanganate AA fibrils loose their congophilia and green birefringence while AL amyloid is resistant (Wright et al. 1977; Van Rijswijk and Van Heusden 1979; Gruys and Timmermans 1979). Although it seems to be a relatively simple technique, in many laboratories there still are considerable problems in performance leading to pseudoresistance in clinically expected AA-amyloidosis.

The present paper describes a retrospective study on biopsy and autopsy material of human patients with various clinical diagnoses (Table 2). First, the results of the potassium permanganate method are compared with the results of immunoperoxidase reactions on the same specimens using six different Munich rabbit antisera against various human amyloid types. These antisera were successfully used in another and larger group of patients with amyloid deposits (Linke et al. 1984a). Secondly, the usefulness of the various antisera for single cases is evaluated. Thirdly, antisera raised against animal AA-proteins were used to investigate whether these antisera could be used to diagnose AA-amyloid in human tissues.

Materials and methods

Tissues. Both biopsy and autopsy material of different organs, obtained from a clinically diverse group of patients, were used (Table 2). All renal specimens were fixed in DuBosq-Brazil solution and embedded in a paraffin bees wax mixture. All other tissues were conventionally fixed in 4% buffered neutral formaldehyde and embedded in paraffin wax. Embedded tissue blocks had been stored at room temperature from a few days up to 13 years. At the time of biopsy or autopsy amyloid was diagnosed with routine histochemical methods. For the present study new sections were cut at 6 μ thickness.

Histochemistry. The alkaline Congo red method according to Stokes (1976) was used as a routine staining procedure to demonstrate amyloid and Wrights modification (Wright et al. 1977) of Romhanyi's potassium permanganate technique (1971 and 1972) to make a first classification into AA- and non-AA amyloid. The tissue sections were dried at 60° C overnight. After deparaffinizing in xylene (twice for 2 min) and rehydration the sections were placed in freshly made 2.5% $KMnO_4$ in 0.15% H_2SO_4 solution (3 min), rinsed in water and cleared in oxalic acid (5%, 5 min). Following thorough washing in distilled water (twice for 5 min) they were gradually brought to ethanol (100%, 1 min), dipped into celloidine (1%) and allowed to air dry. After staining with Stokes (1976) alkaline Congo red (25 min) they were put directly

Table 2. Results of Congo red, potassium permanganate and indirect immunoperoxidase methods

Patient	Sex	Associated disease	Age	Organ	Histochemistry		Immunoperoxidase reaction: anti-human					
					CR	$KMnO_4$	AA	$A\lambda_1$	$A\lambda_2$	$A\lambda_3$	$A\kappa$	AF
BdR	F	RA	64	Jejunum	++	−	+	−	−	−	−	−
				Kidney*	+++	−	+++	−	−	−	−	−
			66	Kidney*	+++	−	+++	−	−	−	−	−
				Heart	+++	−	++	+	+	−	+	−
				Intestine	+++	−	++	+	+	−	−	−
PdB	F	RA	49	Kidney*	++	−	+++	−	+	−	+	−
			55	Duodenum	++	−	++	−	−	−	−	−
ALF	M	RA	65	Kidney	++	−	+++	−	+	−	−	−
ZvR	F	RA	65	Kidney	++	−	+++	−	−	−	−	−
			66	Kidney	+++	−	+++	−	+	−	−	−
PvM	F	RA	55	Rectum	+	−	+	−	−	−	−	−
BdH	F	RA	67	Kidney	++	−	+++	−	−	−	+	−
KEY	M	RA	73	Kidney	++	−	+++	−	−	−	−	−
DGS	F	RA	55	Kidney	+++	−	+++	−	−	−	−	−
				Intestine	++	−	++	−	−	−	−	−
				Skin	++	−	++	−	−	−	−	−
				Aorta	++	−	++	−	−	−	−	−
				pancreas (Vessels)	+++	−	++	−	−	−	−	−
HW	F	RA	67	Kidney	+++	−	+++	−	−	−	−	−
				Heart	+++	−	+++	+	+	−	+	−
				Duodenum	+++	−	++	−	−	−	−	−
				Pancreas:								
				Vessels	+++	−	++	−	−	−	−	−
				Islets	++	++	−	−	−	−	−	−
vTS	F	Idiopathic	41	Kidney	++	−	+++	−	+	−	−	−
HBO	M	Ank. Spondyl.	56	Kidney	++	−	++	−	−	−	−	−
YIL	M	Ank.Spondyl.	42	Kidney	++	−	++	−	−	−	−	−
POM	M	Dermato-myositis	29	Kidney	+++	−	+++	−	−	−	−	−
				Heart	+++	−	+	−	+	−	−	−
				Rectum	++	−	++	−	−	−	−	−
				Prostate:								
				Vessels c.	++	−	++	−	−	−	−	−
				Amylacea	+++	±	−	−	−	−	−	−
FRA	M	Macroglobul. IgM λ	65	Mesenterial tumor	++++	++	−	++	++	+	+	−
BBV	F	Myeloma	63	Synovial membrane	++++	+++	−	++	++	−	++	−
SdN	F	Myeloma IgG λ	54	Kidney	++	+	−	−	++	+	−	−
			55	Kidney	+++	++	−	+	+	+	−	−
				Heart	+++	++	−	+	++	+	−	−
				Esophagus	++	+	−	−	++	+	+	−
				Liver	++	+	−	+	++	+	−	−
				Skin	++	+	−	−	+	+	−	−
vPE	M	Myeloma IgA λ	61	Heart	++	+	−	−	+	+	−	−
RKV	M	Amyloid tumor	39	Larynx	++++	+++	−	+	++	−	−	−
vPP	F	Presenile dementia (Alzheimer)	61	Brain:								
				Plaques	++	+	−	−	−	−	−	−
				Tangles	++	+	−	−	−	−	−	−

* only minimal glomerular involvement

− no staining; ± in general negative, only focal weak staining; + weak; ++ moderate; +++ strong; ++++ very strong

CR = Congo red method according to Stokes[48]; $KMnO_4$ = potassium permanganate method according to Wright[47]; RA = Rheumatoid Arthritis

into ethanol (100%, 1 min), again dipped into celloidine (1%) and air dried before counterstaining with Mayer's haematoxylin (2 min), followed by quick dehydration to xylol and mounting in DPX (BDH Chemicals Ltd. Great Britain). The specimens were examined with conventional light and polarized light microscopy.

Indirect immunoperoxidase method. On all specimens a modification of the indirect immunoperoxidase technique according to Nathrath et al. (1982) was performed as described (Linke et al. 1984b). Six antisera raised against human amyloid proteins obtained from different patients were taken as first antibodies, all prepared in Munich (Linke 1982; Linke et al. 1983; Linke et al. 1984a): anti-amyloid AA (WAL), four antisera against AL-amyloid proteins: anti-Aλ1 (ULI), anti-Aλ2 (HAR), anti-Aλ3 (LOH) and anti-Ak (SIN), and anti-amyloid AF (TIE).

These antisera were raised in crossbred rabbits by subcutaneous injections of alkali-degraded amyloid fibrils in 0.9% NaCl mixed with equal volumes of complete Freund's adjuvant (Difco Lab., USA) and subsequent boostering with purified amyloid proteins. After five monthly injections antibodies were obtained. All antisera were optimally diluted 1:100 in 0.1 M phosphate buffered saline (PBS), pH 7.2. Tissue sections were dried at 60° C overnight for optimal adhesion, deparaffinized, rehydrated, washed in PBS (0.1 M, pH 7.2), treated with H_2O_2 (7.5% in distilled water, 5 min) and again washed in PBS (15 min). Following incubation with normal goat serum (1:10, 30 min) the sections were incubated with the primary rabbit antisera (1:100, 1 h), washed in PBS-Triton X100 (0.005%, 15 min) and again incubated with normal goat serum before applying the second antibody, goat anti-rabbit IgG peroxidase conjugate (Miles Laboratories, Great Britain) 1:800 diluted in 0.1 M PBS, pH 7.2, for 1 h. After washing in PBS-Triton X100 again, peroxidase activity was demonstrated with 3-amino-ethyl carbazol (Grade II, Sigma Chemical Co., MO, USA) at 0.1 mg per ml and 0.0015% H_2O_2, added immediately before use, in 0.1 M PBS, pH 7.2, for 30 min. Following rinsing in tap water and counterstaining with Mayer's haematoxylin the sections were mounted in Kaiser's glycerol gelatine (E. Merck, Darmstadt, FRG). All incubations were performed in a moist chamber at room temperature.

Unlabeled antibody peroxidase-antiperoxidase (PAP) method. On tissue sections of nine patients the PAP-method of Sternberger (1979) was performed. As first antibodies rabbit-anti-human AA (WAL) were applied as described above and a set of antisera raised against animal AA-amyloids, all made in Utrecht (Linke et al. 1984b): anti-canine AA, anti-hamster AA and anti-bovine AA. These antisera were raised in rabbits by intramuscular injections of purified protein AA (Hol et al. 1984) in 1 ml of 0.01 M acetic acid in saline mixed with 1 ml Freund's complete adjuvant. Booster injections every 2 or 3 weeks were of the same dosage, but in incomplete Freund's adjuvant. Three days after the last booster venous blood was collected. The anti-canine AA, anti-hamster AA and anti-bovine AA antisera were optimally diluted (on tissue of the respective species) in 0.05 M Tris HCl buffered saline, pH 7.6, 1:80, 1:50 and 1:80 respectively.

The tissue sections were placed overnight at 60° C, deparaffinized, rehydrated, washed in 0.05 M Tris HCl, pH 7.6, and treated with 7.5% H_2O_2 in methanol (10 min) and then washed in Tris again (10 min). Incubation with normal goat serum (1:20, 15 min) preceded incubation with the primary antiserum (optimal dilutions, 45 min), followed by thorough washing in Tris (30 min). Then again normal goat serum was applied (1:20, 15 min), followed by incubation with goat anti-rabbit IgG Fc (Nordic, The Netherlands), 1:10 diluted in 0.05 M Tris HCl, pH 7.6, for 45 min. After washing in Tris (30 min), incubation with the PAP-complex (1:250, 45 min; Miles Laboratories, England) took place, followed by rinsing in Tris (30 min) and treatment with 0.05% 3,3′-diamino-benzidine tetra HCl (Sigma Chemical Company) and 0.3% H_2O_2 added immediately before use in 0.05 M Tris HCl, pH 8.3, for 15 min to visualize the peroxidase activity. Rinsing in distilled water, counterstaining with Mayer's haematoxylin, dehydration to xylene and mounting in DPX were the final steps. Again all incubations were performed in a moist chamber at room temperature. As controls amyloid containing renal and liver tissue specimens from dog, hamster and bovine cases were stained with the corresponding anti-canine AA, anti-.hamster AA and anti-bovine AA antisera.

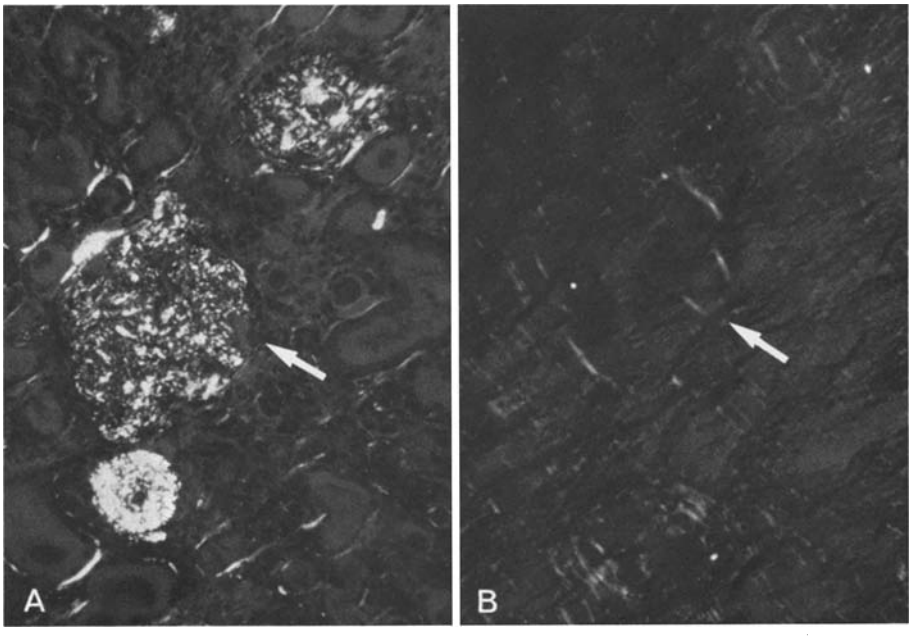

Fig. 1A, B. Glomerular (arrow) and vascular amyloid from a rheumatoid arthritis patient HW (serial sections). **A** Congo red, polarized light, showing the characteristic amyloid birefringence. (×150). **B** After potassium permanganate pretreatment: Congo red birefringence is lost (×150)

Results

Histochemistry

Table 2 gives an overview of the results of the Congo red, potassium permanganate and indirect immunoperoxidase reactions.

After treatment with potassium permanganate both the orange-red staining properties of the amyloid deposits and the green birefringence in polarized light disappeared in all patients with reactive systemic amyloidosis (Fig. 1A and B). In one patient (HW) with rheumatoid arthritis who also developed diabetes mellitus, the pancreatic insular amyloid exhibited undiminished Conge red affinity, in contrast to the systemic amyloid deposits, which showed total sensitivity to potassium permanganate (Fig. 2A and B). In a 29 year old male (POM) who died of a dermatomyositis-like disease, corpora amylacea were found in the prostate and seminal vesicles, which were almost completely permanganate sensitive.

In the immunocyte dyscrasia-related amyloid and in the laryngeal amyloid tumour, potassium permanganate resistance was found with unmistakable apple green birefringence, although the intensity was a little diminished, similar to the sometimes decreased birefringence of other tissue structures (collagen, muscle fibres) (Fig. 3A and B). The amyloid plaques as well as

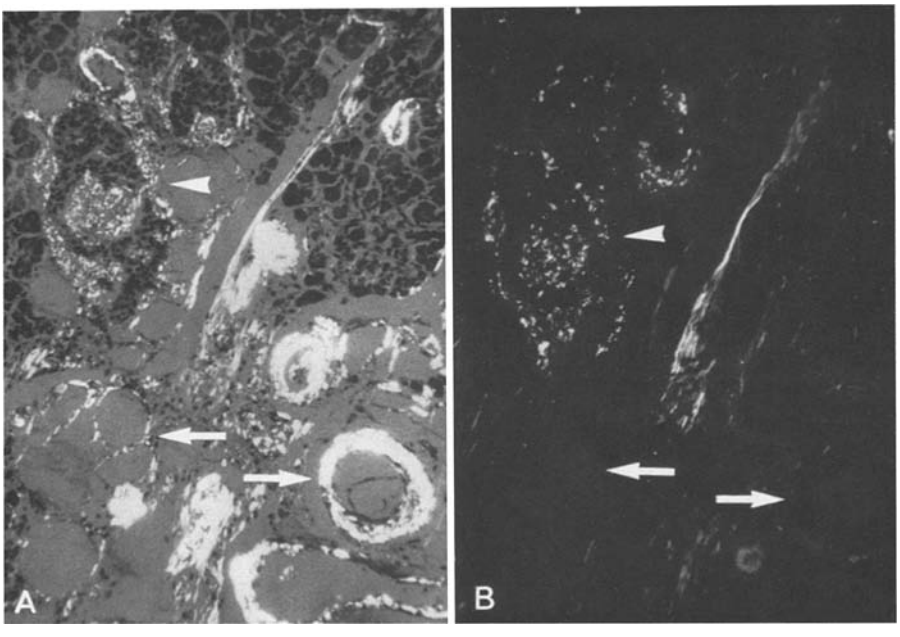

Fig. 2A, B. Pancreatic amyloid from the same patient HW, who had also diabetes mellitus (serial sections). **A** Congo red. Birefringent deposits are found in vessels (*arrow*), adipose tissue (*arrow*) and pancreatic islets (*arrow-head,* ×120). **B** Potassium permanganate, Congo red. Systemic AA-amyloid deposits in vessels and adipose tissue have lost their birefringence in contrast to the unchanged birefringence of localized pancreatic insular AE-amyloid (×120)

the axonal tangles in Alzheimer's disease did exhibit some potassium permanganate resistance.

Indirect immunoperoxidase reaction

The rabbit anti-human AA (WAL) antiserum gave obviously positive staining results in all reactive systemic amyloidosis cases (Table 2, Fig. 4A) and no staining at all in the immunocyte dyscrasia-related amyloidosis cases. The localized forms and the amyloid plaques in Alzheimer's disease were also negative. In some cases proximal tubular epithelial cells stained faintly positive with this antiserum in a granular way, indicating non-selective proteinuria and resorption of AA cross reacting proteins (SAA) by the tubular epithelium.

In renal tissue good congruence was found between the Congo red and immunoperoxidase results, although small paratubular deposits seemed to stain more heavily with the immunoperoxidase method. Sparse reticular amyloid deposits in loose collagen tissue of enteric submucosa were sometimes difficult to distinguish from non-specific background staining in the peroxidase staining method, whilst they were easily found in the Congo red stained sections viewed in polarized light.

The antisera against AL amyloid (anti-Aλ 1–3 and anti-Aκ) gave negative results in most reactive systemic amyloidosis cases, although in some

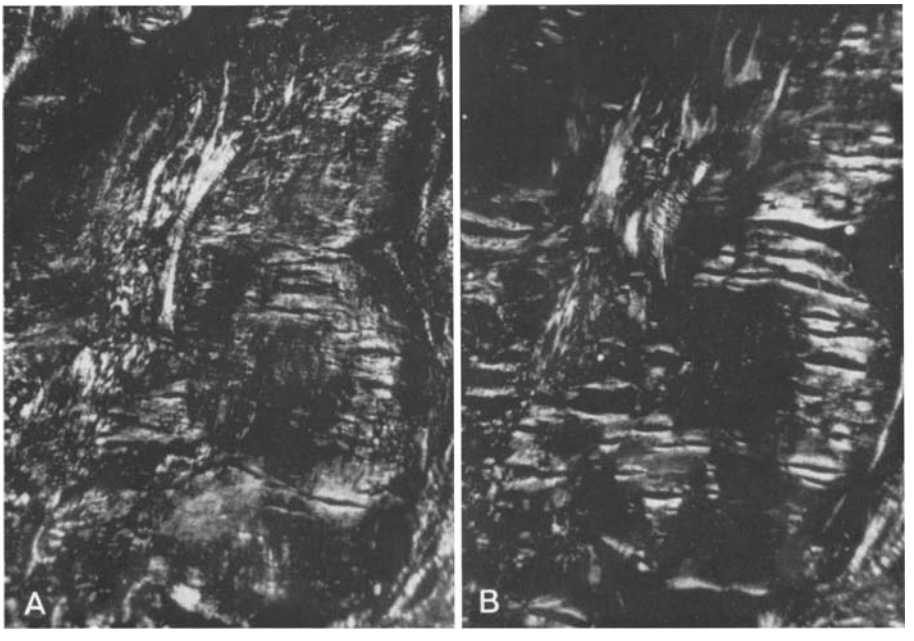

Fig. 3A, B. Amyloid deposits in synovial tissue from a myeloma patient BBV (serial sections). **A** Congo red. The amyloid deposits are birefringent (×120). **B** Potassium permanganate, Congo red. The birefringence is still evident (×120)

cases a few weakly staining amyloid deposits were found, in contrast to the moderately strong positive reactions in immunocyte dyscrasia-derived amyloidosis cases. In one of these cases (BBV), a patient suspected to have myeloma both Aλ and Aκ antisera stained equally positive. With these antisera some plasma cells as well as proximal tubular epithelial cells and tubular casts in patients with proteinuria stained positive, not only in immunocyte dyscrasia-related cases, but also in reactive systemic amyloidosis cases, indicating cross reactivity with both Bence Jones proteins and normal immunoglobulins.

Cases with localized amyloid deposits did not exhibit strong staining reactions with any of the antisera used, with exception of the laryngeal amyloid tumour.

The rabbit anti-AF antiserum gave no reaction with any of the amyloid deposits. Only proximal tubular epithelial cells and tubular casts in patients with proteinuria as well as pericapillary space in the brain tissue of the Alzheimer patient were stained.

PAP method

In general the PAP-method gave more intense staining reactions and a less varying background staining with the anti-human AA serum than the indirect immunoperoxidase staining method (Table 3).

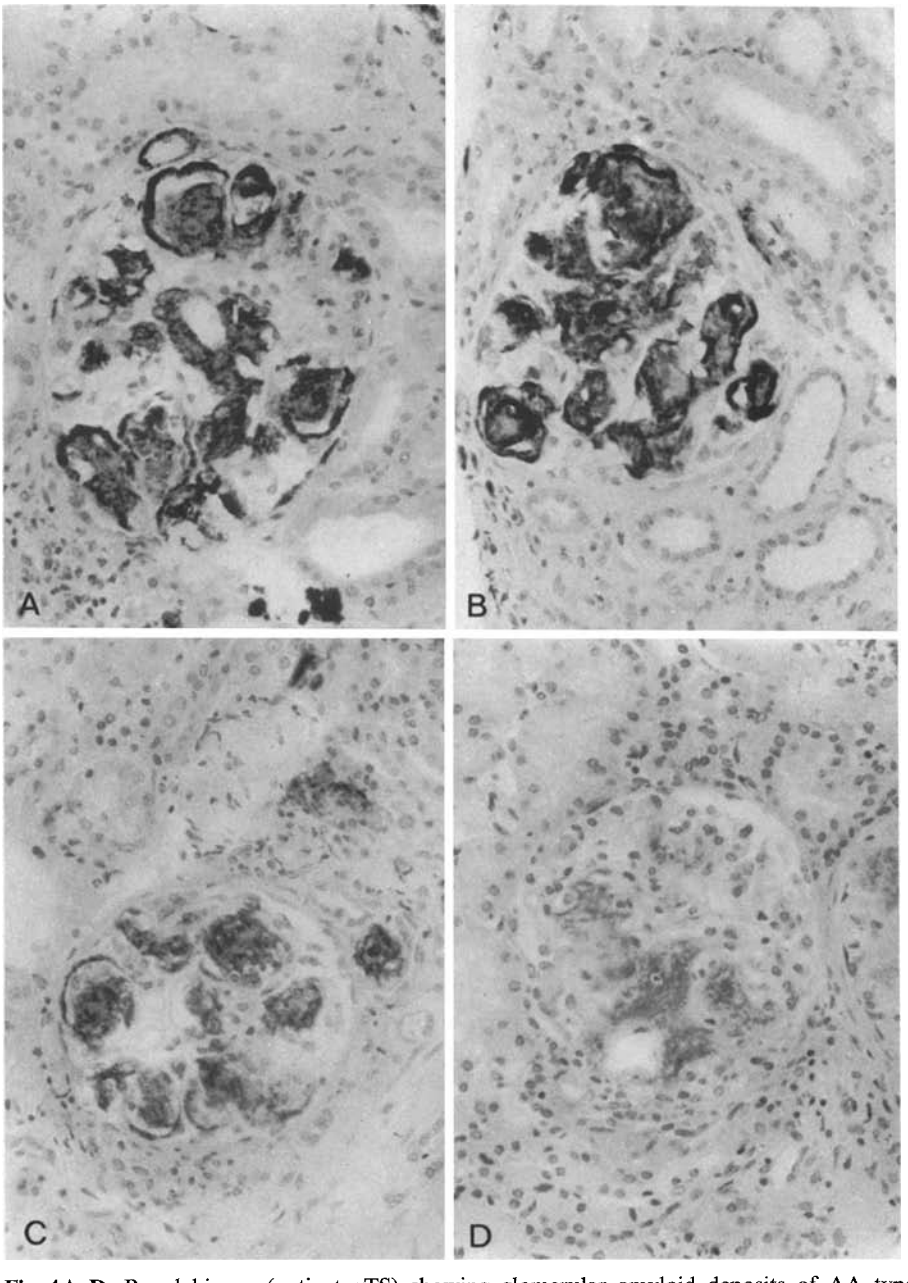

Fig. 4A–D. Renal biopsy (patient vTS) showing glomerular amyloid deposits of AA type. Using the PAP method, serial sections are stained with **A** anti-human AA, **B** anti-bovine AA, **C** anti-hamster AA and **D** anti-canine AA sera (× 260)

Table 3. Results of indirect immunoperoxidase and PAP methods

Patient	Associated disease	Age	Organ	Indirect anti-hum AA	PAP immunoperoxidase methods			
					anti-hum AA	anti-h AA	anti-c AA	anti-b AA
BdR	RA	64	Kidney	+++	++++	++	+	+++
		66	Kidney	+++	++++	+	+	+++
			Heart	++	+++	+	±	++
			Intestine	++	+++	+	±	+++
PdB	RA	49	Kidney	+++	+++	±	+	
ZvR	RA	66	Kidney	+++	++++	++	+	++
BdH	RA	67	Kidney	+++	+++	++	+	+++
DGS	RA	55	Kidney	+++	++++	++	+	+++
HW	RA	67	Kidney	+++	++++	++	+	+++
			Heart	+++	+++	+	±	+++
			Duodenum	++	+++	++	±	+++
			Pancreas:					
			Vessels	++	+++	++	±	+++
			Islets	−	−	−	−	−
vTS	Idiopathic	41	Kidney	+++	++++	+++	++	+++
POM	Dermato-myositis	29	Kidney	+++	++++	+	±	+++
			Heart	+	+++	+	±	+++
			Intestine	++	++	+	±	++
			Prostate:					
			Vessels	++	++	+	±	++
			c. Amylacea	−	−	−	−	−
SdN	Myeloma	55	Kidney	−	−	−	−	−
			Heart	−	−	−	−	−

hum = human; h = hamster; c = canine; b = bovine

Concerning the anti-animal AA sera without any doubt there was cross reactivity with all three antisera, anti-bovine AA, anti-hamster AA and anti-canine AA (Table 3, Fig. 4B, C and D). In the control specimens (Hol and Gruys 1985) both bovine AA amyloid and canine AA amyloid stained very strongly positive (++++) with the homologous antisera and hamster AA amyloid stained strongly positive (+++) with anti-hamster AA antiserum. Between patients the staining intensity with each of the anti-animal AA antisera varied from almost negative to strongly positive.

Discussion

The results show a good correlation between clinical diagnosis with the expected amyloid type and the potassium permanganate reaction. Indeed, all reactive systemic amyloidosis cases exhibited complete sensitivity in contrast to the immunocyte-related cases in which all amyloid deposits showed resistance, although with a less brilliant birefringence. The undiminished congophilia of the pancreatic insular amyloid deposits in the patient with both rheumatoid arthritis and diabetes mellitus indicates a different type of amyloid (Wright et al. 1977; Gruys and Timmermans 1979). This is local-

ized endocrine (AE) amyloid probably derived from insulin or glucagon (Glenner et al. 1974; Cooper 1975; Takahashi et al. 1977; Westermark et al. 1973; Westermark and Wilander 1983), while AA amyloid is present in the other localizations. Such a combination of two different types of amyloid being present in a single patient has been described before (Westermark 1974; Antonutto et al. 1975).

The corpora amylacea found in the prostate and seminal vesicles (Holmes 1977; Westermark et al. 1983; Linke et al. 1984a), (patient POM), presumably consist of a unique fibrillar protein which resembles the AA protein in being potassium permanganate sensitive; however, it is chemically different, since it does not stain with anti-AA serum.

The validity of the potassium permanganate method has been checked against the results obtained with antisera and concordance was found as expected, confirming literature data (Fujihara et al. 1980). The problems of the potassium permanganate method, however, arise in its execution. The potassium permanganate oxidation time is critical ($2^1/_2$–3 min) if it is too long, diminished birefringence of resistant amyloid deposits develops. However, pseudoresistance results if the exposure time is taken too short, e.g. in order to prevent loss of tissue sections. Incubation in a drying stove at 60° C overnight to obtain firm adherance of the tissue sections to the glass slide as well as the use of celloidin may be very helpful in preventing this problem. Moreover, care should be taken in counterstaining with haematoxylin; if this is too heavy small resistant amyloid deposits may become obscured. Finally, the results depend on the skills of the technician.

The conclusion to be drawn is that the potassium permanganate method is useful as a routine staining method if performed well, but some caution must be observed in the definitive interpretation of clinically anomalous results.

Of the immunoperoxidase techniques the anti-protein AA antiserum proved the most useful. Neither the corpora amylacea nor the pancreatic insular amyloid deposits gave positive staining reactions with the anti-AA antiserum, nor did the immunocyte dyscrasia-derived amyloid deposits. All reactive systemic amyloidosis cases, however, showed clearly positive staining reactions, although small amyloid deposits in intestinal submucosa pronded problems in distinguishing them from non-specific background staining.

A difference in staining intensity between kidney and other tissues, e.g. intestine, was found. Differences in fixation i.e. DuBosq Brazil fixative might be responsible.

The anti-AL amyloid antisera gave weakly positive reactions in some cases with obvious AA amyloid. However, these antisera stained some plasma cells in reactive systemic amyloidosis cases as well as tubular epithelial cells and protein casts in patients with proteinuria, indicating cross reactivity with non-monoclonal immunoglobulins. Moreover, amyloid is known to behave like a sponge and various plasma proteins and immunoglobulin deposits have been demonstrated in it (Gruys et al. 1981; Linder and Westermark 1981; Orfila et al. 1983). Both κ and λ light chain cross reacting

antigens have been described on immunohistochemistry of amyloid, but k light chain reactivity predominated in reactive amyloidosis cases, while λ light chains were more abundant in immunocyte dyscrasia related amyloidosis (Husby et al. 1974; Isobe and Osserman 1974; Franklin 1980; Franklin and Gorevic 1980; Orfila et al. 1983). So, if anti-AL amyloid antisera are found to give positive staining reactions in otherwise obvious AA amyloid deposits, this does not prove that minor AL deposits are actually present. Nevertheless negative staining reactions in otherwise suggestive AL amyloidosis (clinical presentation, other staining results) do not exclude AL amyloid, because this is derived from the variable regions of homogeneous light chains, which differ from patient to patient, giving inconsistent staining-reactions. The strong staining reactions in most cases of immunocyte dyscrasia-related amyloidosis may be ascribed to small fragments of constant regions attached to the variable light chain regions of the amyloid fibril, as has been described by amino acid sequence data (Glenner and Page 1976; Glenner 1980; Westermark et al. 1981). However, it may equally well be related to cross reactivity with whole immunoglobulin light chains. The strong positive reaction with both Aλ and Aκ antisera in one patient (BBV) may point in this direction. Whether this disadvantage of rabbit anti-AL sera will be overcome by using monoclonal antibodies is to be tested. If with rabbit AL-antisera positive results are found and negative results with the AA-antiserum, the diagnosis AL amyloid is highly probable, although other non-AA types of amyloid still have to be excluded.

Comparing the indirect immunoperoxidase technique with the PAP method, the latter is expected to be more sensitive because more peroxidase enzymes become linked to one antigen. The results of our study confirmed this hypothesis. The much better results with the PAP method also proved this technique to be more sensitive in paraffin wax sections.

Of the anti-animal AA amyloid antisera, the anti-bovine AA antiserum gave the best cross reactivity in the PAP reactions on human tissues. Only slightly less intensive staining was obtained with this antiserum compared with anti-human AA antiserum. With the other two antisera, anti-canine AA and anti-hamster AA, less cross reactivity and more variability between patients was found, ranging from almost negative to strongly positive. These findings suggest more cross reactivity than described hitherto between human and animal AA-proteins (Linke et al. 1984b). The variability in staining intensity observed may indicate a heterogeneity in human AA amyloid between patients. In animal AA amyloid such variability also has been found (Linke et al. 1984b). However, differences in fixation and age of paraffin blocks may also be taken into consideration. The difference in the previous report that the same anti-bovine AA, anti-canine AA and anti-hamster AA did not stain human AA (Linke et al. 1984b) is probably based on the fact that the more sensitive PAP method has been used in this study.

The intensive staining of human AA amyloid deposits with animal amyloid AA antisera indicates the possibility of using these antisera for the diagnosis of AA amyloid in human tissues.

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