IMMUNOHISTOCHEMICAL IDENTIFICATION AND CROSSREACTIONS OF AMYLOID-A FIBRIL PROTEIN IN MAN AND ELEVEN OTHER SPECIES

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INTRODUCTION

Amyloidosis in man and other vertebrates is a chemically diverse group of diseases caused by generalized, extracellular deposition of approximately 10 nm fibrillar proteins in a β-pleated sheet conformation.

In one class of these amyloidoses, the characteristic major component of the fibrillar deposits is protein AA.* In many cases, the AA-type of amyloid is the result of recurrent inflammation. In certain species, it is either genetically determined or an idiopathic variety (Benditt and Eriksen, 1973; for review, see Glenner, 1980).

Protein AA has been identified in various species including man (Benditt and Eriksen, 1973; Ein, Kimura, Terry, Magnotta and Glenner, 1972; Levin, Franklin and Frangione, 1972; Sletten and Husby, 1974), monkey (Benditt and Eriksen, 1973; Doepel, Glorioso, Newcomer, Skinner and Abrams, 1981; Hermodson, Kuhn, Walsh, Neurath, Eriksen and Benditt, 1972), mouse (Eriksen, Ericsson, Pearsall, Lagunoff and Benditt, 1976; Skinner, Shirahama,

* *Abbreviations:*
AA : Amyloid-A protein.
Aλ : Amyloid of immunoglobulin λ-light chain origin.
Aκ : Amyloid of immunoglobulin κ-light chain origin.
SAA : Serum amyloid-A protein.
SAAL : Low molecular weight component of SAA.
PBS: Phosphate-buffered saline (0·075 molar NaCl and 0·07 molar sodium phosphate, pH 7·2).
Nomenclature recommended at International Conferences in Helsinki, Finland 1974, and in Póvoa de Varzim, Portugal 1979 (Glenner, 1980).

0021-9975/84/030339+18 $03.00/0

Benson and Cohen, 1977), guinea pig (Skinner, Cathcart, Cohen and Benson, 1974), duck (Benditt and Eriksen, 1973; Gorevic, Greenwald, Frangione, Pras and Franklin, 1977), mink (Waalen, Sletten, Husby and Nordstroga, 1980), stone marten (Linke, Geisel, Eulitz and Nathrath, 1980), dog (Gruys, Blewenga and Hol, 1981), cattle (Gruys, 1980; Gruys and Timmermanns, 1979), and hamster (Gruys, Timmermanns and van Ederen, 1979) by amino acid sequence or on the basis of amino acid composition.

In other species, the presence of protein AA was suggested by the distribution of amyloid in organs, its relation to connective tissue structure (Missmahl and Hartwig, 1953) and its sensitivity to oxidation with potassium permanganate (Gruys and Timmermanns, 1979; Wright, Calkins and Humphrey, 1977; Yano, Johnson and Hayden, 1981).

Extensive chemical homologies of protein AA among different species have been described (Gorevic *et al.*, 1977; Waalen *et al.*, 1980), while immunodiffusion (Anders, Natvig, Sletten, Husby and Nordstroga, 1977; Waalen *et al.*, 1980) and immunofluorescence studies did not show any cross-reactivity (Gruys and Timmermanns, 1979), but the enzyme-linked immunoabsorbent assay (Doepel *et al.*, 1981) and the indirect immunoperoxidase technique (Livni, Laufer and Levo, 1980) showed cross-reactivity between the protein of man, monkey and mouse, and between man and mouse, respectively.

Here we describe the identification of AA-type amyloid deposits in tissue sections of man, mouse, hamster, guinea pig, rabbit, cat, dog, mink, stone marten, pine marten, cow, and horse with both intra-species reactions and inter-species cross-reactions of anti-AA antibodies.

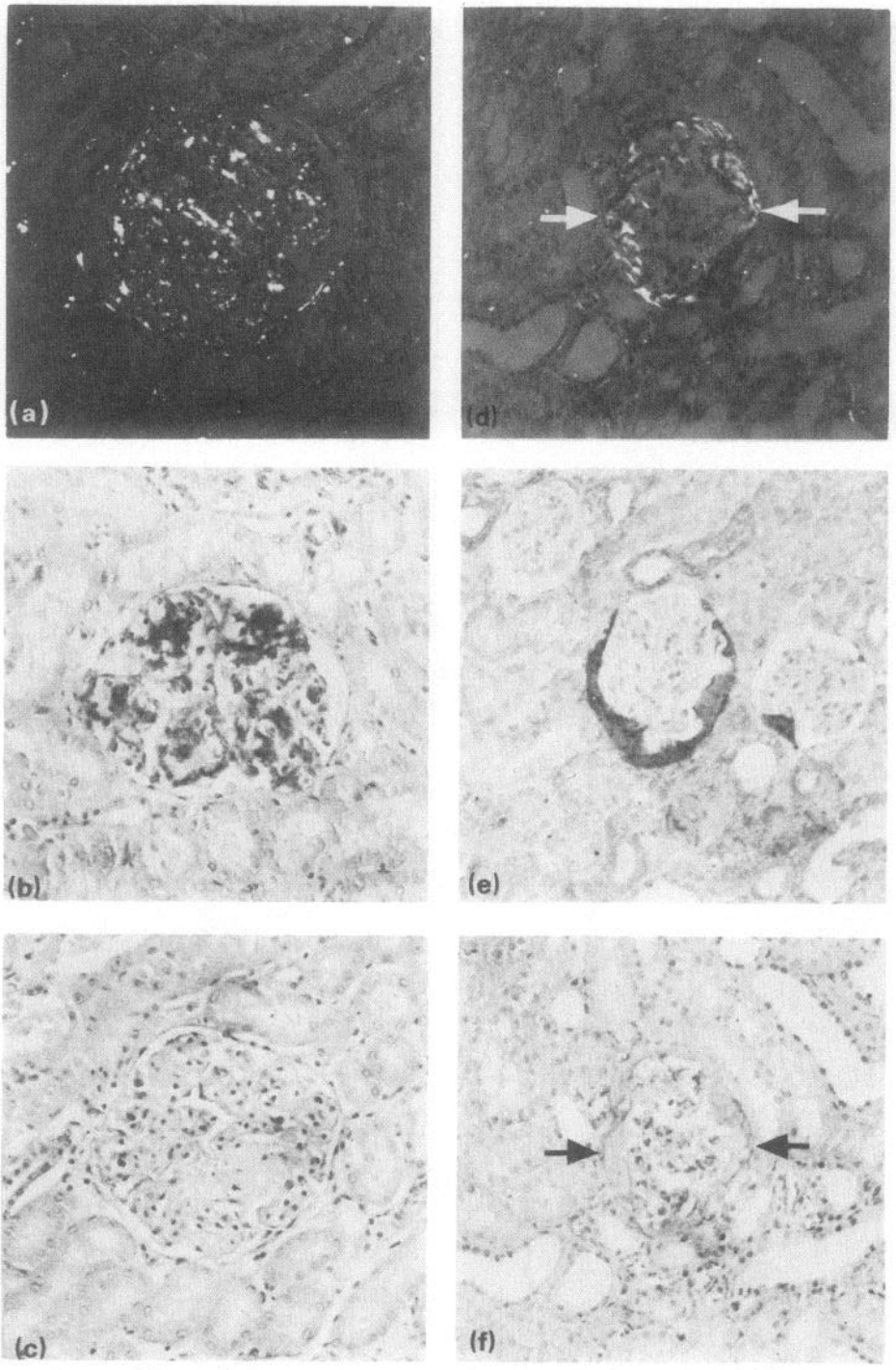
TABLE 1

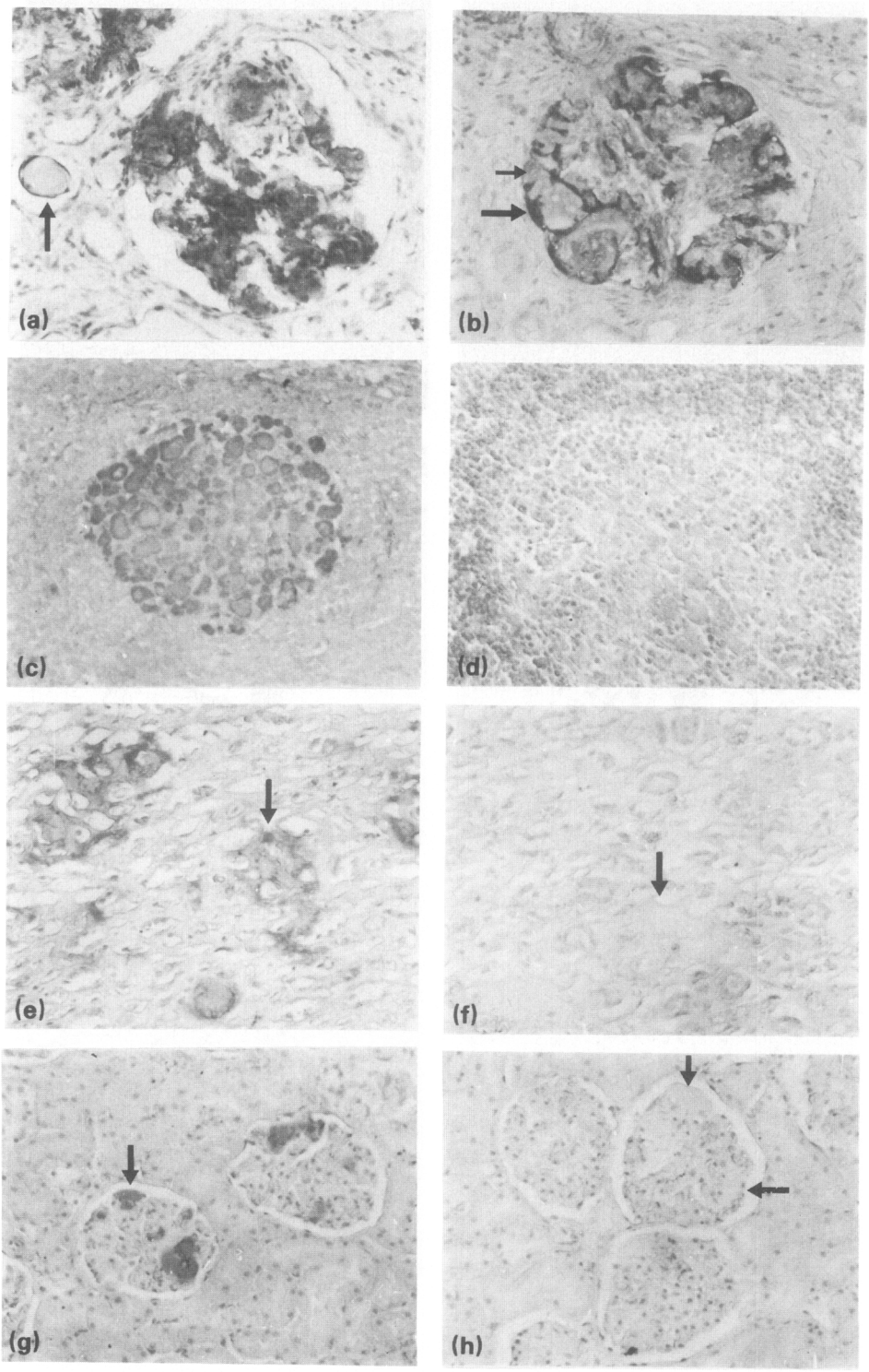
GRADING OF REACTION INTENSITY

Code	*Explanation*	*Examples in Figs 1 and 2*
–	Negative	1(c), (f); 2(f), (h)
l	Slightly more than background	2(d)*
+	Definitely more than background	2(b)**
		2(a) (Cast, see arrow)
+ +	Strong	2(c), (e), (g)
+ + +	Very strong compared with background	1(b), (e); 2(a), (b)**

* Not demonstrable in black and white.
** Centre (+) and periphery of amyloid globule (+ + +).

Fig. 1. Immunohistochemical identification of AA-type amyloid by indirect immunoperoxidase method in dog with antiserum against canine AA, (a)-(c) and in guinea pig with anti-murine AA, (d)-(f). (a) Congo red-stained kidney of dog with glomerular amyloid in polarized light. Bi-refringent areas in glomerulus are bright green in original section. × 180. (b) Adjacent section exposed to a rabbit antiserum against canine AA. All areas within glomerulus identified as amyloid by Congo red staining and green bi-refringence are stained brownish-red, indicating the presence of AA-type amyloid. × 180. (c) Adjacent sections incubated with anti-Aλ (FIS) served as control for section shown in (b); no visible staining. × 180. (d) Congo red-stained kidney of guinea pig with experimentally induced peripheral glomerular amyloid in polarized light. Bi-refringent areas are bright green in original section (between white arrows). × 156. (e) Adjacent section exposed to antiserum against murine AA. Amyloid deposits are stained reddish-brown in original section, thus demonstrating cross-reactivity of protein AA of mouse and guinea pig. × 156. (f) Adjacent section exposed to anti-Aλ (FIS) as control for section shown in (e); no visible staining. Amyloid is located between black arrows. × 156. [See Table 1.]


(a)
(d)
(b)
(e)
(c)
(f)


(a)
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(g)
(h)

MATERIAL AND METHODS

Tissues

Formalin-fixed, paraffin-embedded specimens had been stored for 1 to 17 years. In some cases, only unstained or stained paraffin-sections, in man and the stone marten, cryostat sections also were available. Amyloid was diagnosed on deparaffinized tissue sections (approximately 8 μm thick) by the alkaline Congo red method according to Puchtler, Sweat and Levin (1962), or Stokes (1976) with similar results. The following specimens were examined (Table 2).

Man. Amyloid-containing organs of patients 1 to 4 (FRI, PFL, FIS, and MEV) were obtained at autopsy. The chemical, immunochemical and immunohistochemical identification of amyloid fibril proteins in these specimens has been published elsewhere (Linke, 1982; Linke and Nathrath, 1980).

Mouse. Amyloidosis had been produced in C57/BL6J mice by the induction of osteomyelitis, injections of casein, or injections of casein and complete Freund's adjuvant (Linke, 1969). The material was then stored for 17 years as paraffin blocks or mounted tissue sections.

Syrian hamster (Mesocricetus auratus). Four adult animals: one with amyloid due to old age, one with experimental leishmaniasis and two with amyloidosis following repeated subcutaneous injections of 5 per cent casein solution (Gruys *et al.*, 1979).

Rabbit. Three randomly bred adult rabbits: one acquired amyloidosis during immunization with proteins emulsified in complete Freund's adjuvant (Difco Laboratories, Michigan) and 2 had amyloidosis of unknown aetiology.

Guinea pig. Amyloidosis was induced in adult guinea pigs by casein injections.

Stone marten (Martes foina). Generalized amyloidosis was found accidentally in 3 wild adult stone martens submitted for post mortem diagnosis (Geisel, 1982).

Pine marten (Martes martes). Generalized amyloidosis was found in one wild adult animal submitted for rabies diagnosis.

Mink (Mustela vison). Paraffin-embedded organs from 2 mink: one with induced amyloidosis, one with Aleutian disease (Waalen *et al.*, 1980).

Dog. Nine dogs with generalized amyloidosis included one 6-year-old male bouvier des Flandres (BdF) with prostatitis and a nephrotic syndrome, one 5-year-old male BdF with amyloidosis of unknown aetiology and uraemia, one 6-year-old male golden retriever (GR) with amyloidosis of unknown aetiology and a nephrotic syndrome, one 6-year-old female fox terrier (FT) with cystitis and uraemia (Gruys, Blewenga and Hol, 1981) and 5 dogs with amyloid of unknown aetiology, two of which had uraemia.

Cattle. Six cows: one with glomerular amyloidosis, 2 with idiopathic AA-type (Gruys, 1980; Gruys and Timmermanns, 1979), and 3 with amyloidosis of undertermined type.

Cat (Felis domestica). Four cats: 2 with idiopathic amyloidosis, one 4-year-old male Siamese, one 6-year-old male European shorthair, one cat with amyloid in the islets of Langerhans, one with amyloidosis of unknown aetiology.

Fig. 2. Immunohistochemical cross-reactions of amyloid with various anti-AA antisera by the indirect immunoperoxidase technique in cow (a), (b), horse (c), (d), cat (e), (f), and pine marten (g), (h). (a) Kidney of cow with idiopathic amyloidosis (cow 2, Table 3) stained with anti-bovine AA. Glomerular amyloid is specifically stained reddish-brown in original section (→ = stained cast). (b) Kidney amyloid of cow in (a) stained with anti-dog AA. Glomerular amyloid is specifically stained, with preference for periphery of amyloid lumps (→); centre stained weaker (→). (c) Spleen of horse with follicular amyloid stained with anti-human AA. Amyloid-containing areas are stained brownish-red in original section (horse 3, Table 2). (d) Adjacent section exposed to anti-Aλ (FIS) with border line reaction; section appears without visible reaction product, because the discrete colour is not demonstrable with photography. (e) Kidney of cat with idiopathic amyloidosis. Amyloid deposits in renal medulla are stained with anti-human AA (see →, cat 2, Table 2). (f) Adjacent section exposed to anti-Aλ (FIS) served as control for section shown in (e); no visible reaction product on homogeneous material which was identified as amyloid by Congo red and green bi-refringence (→). (g) Kidney of pine marten exposed to anti-marten AA. Globular structures, identified as amyloid by Congo red staining and green bi-refringence are stained reddish-brown in original section (→). (h) Section adjacent to (g) exposed to anti-Aλ (FIS) served as control. Amyloid-containing areas (→ in one glomerulus) are not stained.

TABLE 2

IMMUNOHISTOCHEMICAL IDENTIFICATION AND CROSS-REACTIONS OF AMYLOID A FIBRIL PROTEINS IN MAN AND ANIMALS BY THE INDIRECT IMMUNOPEROXIDASE METHOD USING ANTISERA AGAINST HUMAN AMYLOID FIBRIL PROTEINS. THE ENCLOSED AREAS MARK HOMOLOGOUS (HEAVY BOXES) AND HETEROLOGOUS (LIGHT BOXES) REACTIONS

Tested tissue					*Antisera against*						
					(1)* *AA/Man*					*Aλ/Man*	*Aκ/Man*
					MED	*BUC*	*WEB*	*FRI/UPS*	*FRI/UPS*	*FIS*	*SIN*
Amyloid† *(species)*	*No.*	*Diagnosis*	*Organ and type of section‡*		(2) *Rabbit* (3) *1:100*	*Rabbit* *1:50*	*Rabbit* *1:100*	*Sheep* *0·01¶*	*Chicken* *1:1000*	*Rabbit* *1:1000*	*Rabbit* *1:100*
Man	1	Crohn's disease	Kidney	c/p	+++§	+++	++	++	++	–	–
	2	Rheum. arthr.	Kidney	c/p	++	++	++	++	++	–	–
	3	Idiopathic A.	Liver	c	–	–	–	–	–	++	–
	4	Multiple Myeloma	Duodenum	p	–	l	–	l	l	–	+++
Mouse	1	Exp. ostcomyelitis	Kidney	p	+	–	–	++	+	–	–
			Spleen	p	+	–	–	+	–	–	–
			Liver	p	++	+	l	++	+	–	–
	2	Casein injection	Kidney	p	+	–	l	l	–	–	–
	3	Cas. + Frd's.Adj.Inj.	Liver	p	++	+	+	+	+		
	4	Cas. + Frd's.Adj.Inj.	Liver	p	++	++	+	–	–		
Hamster	1	Old age	Spleen	c	–	–	–	l	+	–	–
	2	Leishmaniasis	Liver	p	–	–	–	–	–	–	–
	3	Casein injection	Liver	p	–	–	–	–	l	–	–
	4	Casein injection	Liver	p	–	–	–	l	+	–	–
Guinea pig		Casein injection	Kidney	p	++	++	+	++		lf††	–
Rabbit	1	Immunization	Kidney	p	+	–	l	+	+	–	–
	2	Idiopathic A.	Liver	p	–	–	–	–	l	–	–
	3	Idiopathic A.	Liver	c	–	–	–	l	l	l	–
Stone marten	1	Idiopathic A.	Kidney	c/p	–	–	–	+	+	l	–
	2	Idiopathic A.	Kidney	p							–
	3	Idiopathic A.	Kidney	p							–
Pine		Idiopathic A.	Liver	p	–	–	–	–	l	–2h	–

Mink		1	Endotoxin inj.	Liver	p	–	–	–	–	+	–	–
		2	Aleutian disease	Spleen	p	–	–	–	–	l	–	–
Dog	BdF††	1	Prostatitis	Kidney	p	–	–	–	–	+	–	–
	BdF	2	Idiopathic A.	Kidney	p	–	–	–	+	+	– 1c	–
	GR	3	Idiopathic A.	Kidney	p	–	–	–	–	l	–	–
	FT	4	Cystitis			–	–	–	–	+	–	–
	WD	5	nd	Kidney	p				–	+	–	–
		6	nd	Kidney	p						–	
		7	nd	Kidney	p						–	
	P	8	Uraemia	Kidney	p						–	
	MP	9	Uraemia	Kidney	p						–	
Cat		1	Idiopathic A.	Kidney	p	+	+	+	+	+	–	–
		2	Idiopathic A.	Kidney	p	+ +2e	–	–	+	+	–2f	–
		3	nd	Kidney	p	+ +					–	
		4	Old age	Pancreas	p	–	–	–	–	–	–	–
Horse		1	Idiopathic A.	Kidney	p	+ +	l	+	+ +	+ +	–	–
		2	Immunization	Liver	p	+	–	–	+ +	l	–	–
	TH	3	nd	Spleen	p	+ +2c					l‖2d	
		4	Idiopathic A.	Liver	p	+ + +	+	+ +	+	–	–	–
		5	nd	Liver	p	+ + +	+ +	+	+ +	l	–	–
		6	nd	Liver	p	+	+	+ +	–	–	–‖	–
Cow		1	Idiopathic A.	Kidney	p	+	–	–	–	+	l‖	+
		2	Idiopathic A.	Kidney	p	–	–	–	–	–	–	–
		3	nd	Kidney	p	+	+ +	+	l	–	–	–
		4	nd	Kidney	p	+ +	+ +	+ +	+	–	–	–
		5	Glomerul. amyloid.	Kidney	p	+ +	+ +	+	–	–	–‖	–

*(1) Amyloid fibril proteins (type, species, patient's initials; (2) Produced in (species); (3) Dilution.
†As defined by the alkaline Congo red staining and green bi-refringence.
‡c/p=Cryostat and paraffin section.
§Grading of reaction: –, l, +, + +, + + + (see Table 1).
¶ Purified specific antibody (mg/ml).
‖After absorption of antiserum with human or bovine serum (see Results).
** Abbreviation of breed: BdF, GR, FT, (see "Material and Methods"). WD=wire-haired dachshund; MP=miniature Pomeranian; TH=Trakehner horse
††Numbers refer to Fig. 1(a)–(f) and 2(a)–(h).
nd No data available.

Horse. Six horses: 5 with generalized idiopathic, and one with immunization-induced generalized amyloidosis complicated by hepatorrhexis.

Control tissues were taken from human non-AA amyloid, i.e., Aλ (FIS) and Aκ (MEV) (Linke and Nathrath, 1980).

Antisera

Antisera used in these studies are listed in Tables 2 and 3. The immunized animals were crossbred rabbits and merino sheep.

Rabbit anti-human AA sera. The preparation of anti-AA (MED) and anti-AA (BUC) has already been described elsewhere (Linke, 1981; Linke, Sipe, Pollock, Ignaczak and Glenner, 1975). Anti-AA (FRI), anti-AA (WEG) and a combination of anti-AA (FRI + UPS) were prepared as described for anti-AA (BUC). Anti-human AA was absorbed with normal human serum coupled to cyanogen bromide-activated Sepharose 4B (Linke, 1981).

Chicken anti-human AA (UPS + FRI) serum. This serum was prepared by injecting 2·5 mg of guanidine-HCl denatured amyloid fibrils into the breast muscle of a 5-month-old Leghorn chicken, followed by injections of 1·5 mg and 0·5 mg of purified protein AA, 3 and 4 months later. The antigen was homogenized in 0·5 ml of 0·9 per cent sterile NaCl and 0.5 ml of complete Freund's adjuvant (Difco Laboratories, Michigan). Blood was drawn from the cubital vein 1 week after the last booster injection.

Sheep anti-human SAAL. This was prepared and tested for specificity as described (Linke, 1981).

Rabbit and sheep anti-murine AA. These were raised as reported for anti-AA (BUC) (Linke, 1981). Murine amyloid AA was prepared from amyloid-containing spleens and livers of 40 mice (amyloidosis was induced with complete Freund's adjuvant and casein (Linke, 1969) and extracted (Pras, Zucker-Franklin, Rimon and Franklin, 1969). Amyloid fibrils from the spleen were dissolved in 5 M guanidine-HCl and separated on Sephadex G-100 (Glenner, Harbaugh, Ohms, Harada and Cuatrecasas, 1970). The major retarded peak was approximately 10,000 Daltons.

Rabbit and sheep anti-stone marten amyloid fibril protein. This was prepared as described (Linke *et al.*, 1980).

Rabbit anti-hamster AA, anti-canine AA, and anti-bovine AA antisera. Dutch-Lotharingen crossbred rabbits were given intra-muscular injections of 1 mg of purified protein AA (Gruys and Timmermanns, 1979) in 1 ml of 0·9 per cent NaCl with 0·01 M acetic acid emulsified in 1 ml of complete Freund's adjuvant (Difco Laboratories, Michigan), at two different sites. Booster injections 4 and 12 weeks later were of the same dosage, but in incomplete Freund's adjuvant. One week after the last injection blood was drawn from the ear vein. Anti-canine and -bovine AA antiserum were used as such, while anti-hamster AA was absorbed with normal hamster liver homogenate.

Control antisera. This included rabbit anti-Aλ (FIS) and rabbit Anti-Aκ (SIN) (Linke, 1982, Linke and Nathrath, 1980).

Peroxidase-conjugated immunoglobulins for the indirect immunoperoxidase technique are described below.

Immunodiffusion

Two-dimensional immunodiffusion was carried out in 1·0 per cent Seakem-agarose (Type ME, MCI, Biomedical Division of Marine Colloids INc., Rockland, Md/USA) in pH 8·6 barbital buffer (ionic strength, 0·03) and 0·02 per cent NaN_3 (Merck, Darmstadt, West Germany). The antigen concentration was 0·1 to 0·2 mg per ml of the same solvent or PBS. The antisera were used either as such or concentrated 2 to 3 times for optimal precipitation with the immunizing antigen.

Indirect immunoperoxidase method

The method used was based on that described before (Nathrath, Wilson and

Trejdosievicz, 1982). In brief, after optimizing the signal-to-noise ratio, the first antisera were used at dilutions of up to 1 in 200 (optimal dilutions are given in Tables 2 and 3) and the second antibody at 1 in 200 to 1 in 400 in PBS. Controls included rabbit antiserum against a non-AA protein, omitting either the first or the second antibody or both.

Tissue sections were dried at 70 °C for several hours, deparaffinized, treated with 7·5 per cent H_2O_2 for 10 min and incubated with 3 to 10 per cent normal serum (in PBS) of the species in which the second antiserum was prepared. Incubation of the first antibody (30 min) was followed by washing in PBS-B [B is 0·005 per cent Brij 35 (polyoxyethylene lauryl ether); Merck-Schuchart, Hohenbrunn, West Germany], incubation with the IgG-peroxidase conjugate (Miles Research Products GmbH, Frankfurt, West Germany) for 30 min. Peroxidase activity was visualized with 3-amino-ethyl carbazol (grade II, Sigma Chemical Comp., Taufkirchen, West Germany) and H_2O_2 (E. Merck, Darmstadt, West Germany) at 0·1 mg per ml and 0·0015 per cent in 0·1 molar pH 7·4 barbital buffer, respectively. Counter-stain was Mayer's haemalum, blued in tap water and mounted in Kaiser's glycerol gelatine (E. Merck). Cryostat sections were treated similarly after fixation in acetone (E. Merck) for 10 min.

Grading of peroxidase staining

The immunohistochemical staining intensity of the amyloid-containing areas was graded by two independent investigators. When the gradings differed the experiments were carried out again and evaluated similarly. The mode of grading is shown in Table 1 and illustrated in Figs 1 and 2. The mean values of several experiments are presented in Tables 2 and 3. Cross-reactivity was considered significantly strong when at least one-half of the tested animals of one species showed a reaction.

Sensitivity to oxidation and documentation

Specimens of all species were treated with the potassium permanganate procedure according to the modification of Romháni's method (Romháni, 1972) by Wright *et al.* (1977). Photography and documentation were carried out as described by Linke (1982).

RESULTS

Immunodiffusion Analysis

A total of 12 purified amyloid fibril proteins, i.e. human AA (WEB, BUC, FRI, UPS, PFL), human Aλ (FRD), human Aκ (MEV) and purified animal AA (mouse, stone marten, hamster, cow, dog) were investigated by double diffusion with 15 different antisera against purified amyloid fibril proteins (see Tables 2 and 3 for details of antisera), amounting to 180 different combinations.

Immunoprecipitation lines were obtained only with protein AA of that species against which the antibodies had been raised, with three exceptions: anti-murine AA prepared in rabbits inconsistently showed a faint precipitation line with several human AA preparations, anti-human AA prepared in chickens showed a similar precipitation line with murine AA, and the antiserum against hamster AA was so weak that it did not precipitate, although it stained amyloid in tissue sections (Table 3).

Indirect Immunoperoxidase Method

General observations. Cryostat and/or paraffin-embedded tissue sections from 47 cases of amyloidosis in 12 mammalian species were investigated with 16

TABLE 3

IMMUNOHISTOCHEMICAL IDENTIFICATION AND CROSS-REACTIONS OF AMYLOID-A FIBRIL PROTEINS IN MAN AND ANIMALS BY THE INDIRECT IMMUNOPEROXIDASE METHOD USING ANTISERA AGAINST AMYLOID FIBRIL PROTEINS FROM ANIMALS. (FOR EXPLANATIONS SEE TABLE 2.)

					Antisera against						
	Tested tissue				*AA/ Mouse Rabbit 1:100*	*Sheep 1:100*	*AA/ Hamster Rabbit 1:50*	*AA/ Marten Rabbit 1:100*	*Sheep 1:100*	*AA/ Dog Rabbit 1:200*	*AA/ Cow Rabbit 1:100*
Amyloid† (species)	*No.*	*Diagnosis*	*Organ and type of section‡*								
Man	1	Crohn's disease	Kidney	c/p	+	−	−	−	−	−	−
	2	Rheum. arthr.	Kidney	c/p	+	−	−	−	−	−	−
	3	Idiopathic A.	Liver	c	−	−	−	−	−	−	−
	4	Multiple Myeloma	Duodenum	p	−	−	−	l	−	−	−
Mouse	1	Exp. osteomyelitis	Kidney	p	+ + +	+ +	−	−	−	−	−
			Spleen	p	+ +	+	−	−	−	−	−
			Liver	p	+ + +	+ +	−	−	−	−	−
	2	Casein injection	Spleen	p	+ +	+	−	−	−	−	−
	3	Cas. + Frd's.Adj.Inj.	Liver	p	+ + +	+	−	−	−	−	−
	4	Cas. + Frd's.Adj.Inj.	Liver	p	+ + +	+ +	−	−	−	−	−
Hamster	1	Old age	Spleen	c	+	+	+ +	−	−	−	l
	2	Leishmaniasis	Liver	p	l	+	+ + +	−	−	−	−
	3	Casein injection	Liver	p	−	−	+ +	−	−	−	−
	4	Casein injection	Liver	p	−	−	+ +	−	−	−	−
Guinea Pig		Casein injection	Kidney	p	+ + + le	+	−	−	l	−	−
Rabbit	1	Immunization	Kidney	p	−	l	−	−	−	−	−
	2	Idiopathic A.	Liver	p	−	l	−	−	l	−	−
	3	Idiopathic A.	Liver	c	+	l	−	−	−	−	−
Stone marten	1	Idiopathic A.	Kidney	c/p	−	l	−	+ + +	+	−	−
	2	Idiopathic A.	Kidney	p	−	l	−	+ + +	+ + +	−	−

Pine marten		Idiopathic A.	Liver	p	–	–	–	+ + 2g						
Mink	1	Endotoxin inj.	Liver	p	–	+	+	+	+ +	+	–			
	2	Aleutian disease	Spleen	p	–	1	+ +	+ +	+ + +	+	–			
Dog BdF**	1	Prostatitis	Kidney	p	–	1	+	+	–	+ + +	1			
BdF	2	Idiopathic A.	Kidney	p	+	+	+	+	–	+ + + 1b	+			
GR	3	Idiopathic A.	Kidney	p	+	1	–	–	–	+ + +	+			
FT	4	Cystitis			–	+	+	–	–	+ + +	–			
WD	5	nd	Kidney	p	+	1	–	1	–	+ + 1	–			
	6	nd	Kidney	p						+ +				
	7	nd	Kidney	p						1 1				
P	8	Uraemia	Kidney	p						1 +				
MP	9	Uraemia	Kidney	p						+ + +				
Cat	1	Idiopathic A.	Kidney	p	+	–	–	–	–	–	–			
	2	Idiopathic A.	Kidney	p	1	1	–	–	–	1	–			
	3	nd	Kidney	p	+					+ +				
	4	Old age	Pancreas	p	–	–	–	–	–	–	–			
Horse	1	Idiopathic A.	Kidney	p	1	+	+	–	–	1	–			
	2	Immunization	Liver	p	–	–	1	–	–	1	–			
TH	3	nd	Spleen	p										
	4	Idiopathic A.	Liver	p	–	–	–	–	–	1	–			
	5	nd	Liver	p	+	–	+	1	–	1	–			
	6	nd	Liver	p	+ +	–	–	–	–	–	–			
Cow	1	Idiopathic A.	Kidney	p	–	–	+ +	–	–	+ +	+ + +			
	2	Idiopathic A.	Kidney	p	–	+	+	–	–	+ + 2b	+ + + 2a			
	3	nd	Kidney	p	+ +	+	+	–	–	+	+ + +			
	4	nd	Kidney	p	+ +	+	+ +	+	1	+	+ + +			
	5	Glomerul. amyloid.	Kidney	p	+ +	+	+	1	1	1	+ + +			

** For abbreviations of breeds please see Materials and Methods.

antisera directed against amyloid fibril (or related) proteins. The results of approximately 2000 experiments are summarized in Tables 2 and 3. Representative results are illustrated in Figs 1 and 2.

When Congo red and immunohistochemically stained areas were compared, both staining methods of amyloid were found to be congruent. In two instances, however, the immunohistochemical staining was found in areas without amyloid: (1) in liver cells in hamster, mouse and horse situated close to the Glisson's triad containing stained fine granular material, indicating the cells of AA-precursor synthesis (Selinger, McAdam, Kaplan, Sipe, Vogel, and Rosenstreich, 1980) and (2) staining of both renal tubular casts and tubular cells in stone marten (Linke *et al.*, 1980) dog, cat, horse and cow, indicating glomerular filtration and reabsorption by tubular cells of the AA-precursor SAAL in non-selective proteinuria (Linke, von Giese, Bohle, Thomas, Grüner, Riethmüller and Beckh, 1980).

The specificity of the immunohistochemical reactions in man, mouse, stone marten, hamster, cow, and dog was demonstrated by the following criteria. (a) The staining in a given species was significantly reduced or completely abolished when the antisera were absorbed with the corresponding antigen. (b) No reaction was observed when the first or the second antibody was omitted. (c) No reactions were observed using antisera to non-AA types of amyloid, except for a few borderline reactions. (d) Anti-AA antisera did not stain non-selectively, but in distinct patterns, which strongly suggest specificity (see Tables 2 and 3).

Differences in staining intensity were also noted which varied from animal to animal or sometimes from organ to organ, and even within a given amyloid deposit. In the latter case the edges of the amyloid deposits were sometimes stained more strongly than the centre [Fig. 2(b)]. In addition, when compared with Congo red staining, immunohistochemical staining was considerably more sensitive. Many very small amyloid deposits, which could have escaped identification by polarization microscopy (after Congo red staining), were clearly identified. Moreover, neither the use of cryostat sections as tested in man and stone marten amyloid (Tables 2 and 3), nor pronase digestion (0·1 mg per ml pronase from *Streptomyces griseus*, type V [Sigma] in PBS for 3 h) improved the staining.

Immunohistochemical identification of amyloid A fibrils. All amyloid deposits were strongly stained with antisera against AA from a member of the respective species (homologous reaction, see heavy boxes in Tables 2 and 3). In addition, Congo red staining and immunohistochemical staining were congruent (see above).

Immunohistochemical crossreactions of anti-human AA with animal amyloids. All five antisera, each prepared in a different animal against protein AA of a different human patient, identified protein AA from all patients similarly well (see above). However, when these antisera were applied to amyloid of animals, various cross-reactions were observed, with reactions weaker than in the homologous situation. Amyloid deposits in one species did not necessarily react equally well with all five antisera. For example, rabbit-anti-human AA (MED), cross-reacted strongly with amyloid of mouse, guinea pig, cat (generalized form), horse, and cow, while anti-AA (BUC) and (WEB), although having a very similar reaction pattern, reacted more restrictedly (see Table 2). The sheep-anti-human AA (FRI/UPS) showed a similar reaction pattern to the rabbit antiserum, but it also reacted with

rabbit and stone marten amyloid. Similar results were obtained with a sheep antiserum against human SAAL (KEL) which reacted also with dog and particularly strongly with rabbit amyloid (results not shown).The widest spectrum of cross-reactivities was found with a chicken-anti-human, AA which reacted with amyloid in all species tested, although it was not equally strong with all animals within one particular species. Only one of five cows cross-reacted, while one horse showed strong reactivity and the other two only borderline reactivity. More than half of the subjects of each of all other species reacted.

Immunohistochemical cross-reactions with antisera to animal AA. Similar to the anti-human AA sera, the antisera raised against amyloid of animals reacted strongly with the respective species (Table 3, heavy boxes), but varied in their cross-reactivity. Rabbit-anti-murine AA cross-reacted with amyloid of man, guinea pig [+ + +, Fig. 1(e)], dog, cat, horse, and cow. In contrast, sheep-anti-murine AA did not stain human, feline or equine amyloid but did stain hamster, guinea pig, rabbit, stone marten, canine, and bovine amyloid. Rabbit-anti-hamster AA stained amyloid from mink, dog, horse, and cow. Rabbit-anti-stone marten AA stained pine marten [Fig. 2(g)], mink and dog amyloid. When prepared in sheep, anti-marten AA reacted only with mink amyloid. Rabbit-anti-canine AA cross-reacted with amyloid of mink, cat, and cow; the reaction with horse amyloid was of borderline strength. The cross-reactivity of rabbit-anti-bovine AA was even more limited. Although it reacted very strongly against all 5 cows tested [Fig. 2(a), Table 3] it cross-reacted only with amyloid of one other species, i.e. dog. It should be noted that cross-reactivities were mutual in only 2 relations: mouse-man and dog-cow. In contrast, hamster amyloid was identified in tissue sections with anti-human AA and anti-mouse AA, while anti-hamster AA did not stain human or murine amyloid. In addition, the very strong antiserum against bovine AA did not stain human, murine or hamster AA, although antisera against some of these amyloids strongly stained bovine AA in tissue sections.

While all tested animal amyloids showed some degree of cross-reactivity with at least one anti-AA reagent, feline pancreatic islet amyloid did not react with any of the antisera.

Sensitivity to Oxidation

Potassium permanganate oxidation of amyloid deposits was found in all species, except in feline pancreatic amyloid. Most animals showed a reduction in Congo red staining and green bi-refringence of up to 90 per cent but in the mouse it was about 70 per cent and 20 per cent in one horse, while another horse showed a reduction of about 80 per cent.

DISCUSSION

The results show that amyloid, as identified in many species by Congo red staining and green bi-refringence, can also be characterized by immunological methods. The 12 antisera to purified amyloid protein AA of 5 species raised in rabbits, sheep or chickens, reacted specifically and strongly with the amyloid deposits not only in the animal from which the immunizing amyloid was isolated and with amyloid of members of the same species but also cross-reacted with

amyloid of other species, although to a lesser extent. Thus, by the criterion of interspecies cross-reactivity, which is based on common or similar antigenic epitopes, AA-type amyloid fibrils have been shown for the first time to be present in rabbit, pine marten, cat (generalized form) and horse. In addition, the presence of AA-amyloid in hamster, guinea pig, stone marten, mink, dog and cow (for references see "Introduction") has been confirmed immunohistochemically with anti-AA antibodies and the immunoperoxidase method on formalin-fixed paraffin sections.

According to known amino acid sequence data, cross-reactions among AA-proteins of different species can be expected, particularly when ultra-sensitive methods are used, as represented, for instance, by the immunoperoxidase techniques (Nakane and Pierce, 1966; Sternberger, 1979).

The AA-type is the most common form in mammalian amyloidosis based on immunohistochemical data and permanganate sensitivity. Chemically different types of amyloid have been identified in man (Glenner, 1980) and possibly in the mouse (Higuchi, Matsumara, Honma, Takeshita, Hashimoto, Hosokawa, Jasuhira and Takeda, 1983). The pancreatic islet amyloid of the cat is also of non-AA nature, since it is non-reactive with anti-AA antisera and is permanganate resistant [this confirms the results of Yano, *et al.* (1981)], in contrast to the generalized feline amyloidosis which bound anti-AA antibodies (see above).

The method used was demonstrated to be specific and reliable by the use of purified and partially sequenced proteins and by the results of the control experiments. In addition, this method had been shown previously to be valid for identifying and classifying amyloid in tissue sections in man (Livni *et al.*, 1980; Fujihara, Balow, Costa and Glenner, 1980, Linke and Nathrath, 1980, Linke, 1982), mouse (Fujihara *et al.*, 1980; Livni *et al.*, 1980) and stone marten (Linke *et al.*, 1980).

In animals, AA-type amyloidosis is associated with naturally occurring or experimentally induced forms of inflammation. When inflammation could not be found, the presence of an idiopathic amyloidosis was assumed, as for instance in the spontaneous amyloid in the aged hamster and other cases (see Tables 2 and 3). The high incidence of amyloidosis in the stone marten (10 per cent of submitted animals) may suggest a genetic factor, in particular, since detailed comparison in martens with and without amyloidosis did not reveal any differences such as the presence of inflammation or parasites (Geisel, 1982; Geisel, Krampitz and Pospischil, 1979). Also, in man, amyloidosis of the AA-type occurs in association with recurrent or chronic forms of inflammation, with familial Mediterranean fever, without an apparent predisposing disease, i.e. as an idiopathic variety (reviewed by Glenner, 1980), or with the Muckle-Wells syndrome (Linke, Heilmann, Nathrath and Eulitz, 1983).

The extensive variability of protein AA in immunohistochemical reactions arises from the fact that it is a small molecule of 6000–14 000 Daltons with few antigenic determinants. Some of these may be hidden in the amyloid fibril or altered by preservation techniques. Also, variations in size (Lian, Benson, Skinner and Cohen, 1975), charge (Linke *et al.*, 1975; Westermark, 1982) and amino acid sequence (Gorevic, Levo, Frangione and Franklin, 1978) have been described. Antigenic epitopes relevant for binding may also be altered by mutations based on evolutionary distance. These variations may be detected by

antisera against protein AA of different species prepared in a suitable animal. These considerations, as derived from immune reactions with fibrillar proteins in tissue sections, are consistent with immunochemical results obtained on small soluble proteins, e.g. human haemoglobin and cytochrome C of different species, as reviewed by Reichlin and Noble (1977). It was shown that immune reactions towards a single antigenic determinant reflect primarily chemical similarity and not evolutionary distance. Only when, for instance, proteins are compared with antisera directed against many antigenic epitopes and thus averaging the discontinuity of mutation-induced changes of single antigenic determinants may the strength of the cross-reaction reflect evolutionary distance. In addition, an immunized animal is tolerant towards exposed regions of a protein which are identical to those of its own homologous protein. Thus, rabbit amyloid could in general only be stained by antisera from animals other than the rabbit. Cross-reactions indicate similarity between immunizing antigen with those parts of the AA-molecule which are recognized as foreign by the immunized animal. Cross-reactions may be reciprocal as in the mouse-man relationship (see "Results") or non-reciprocal as in the man-cow relationship. The latter case demonstrated that rabbit antisera raised against human AA cross-reacted with bovine AA and these bovine epitopes were unable to induce antibodies in rabbits which cross-react with human AA. Immunochemical differences between protein AA of various species as shown by our data could be further elaborated by detailed chemical and antigenic studies mapping antigenic determinants, possibly assisted by monoclonal antibodies against AA-proteins.

SUMMARY

Antisera were prepared in rabbits, sheep or chicken against purified amyloid fibril protein AA from man, mouse, stone marten, dog, cow and hamster. These antisera were tested by immunodiffusion against all purified antigens and applied to tissue sections containing amyloid from man, mouse, hamster, guinea pig, rabbit, cat, dog, mink, stone marten, pine marten, cow and horse. The binding of the antibodies to amyloid in tissue sections was assessed by the indirect immunoperoxidase method.

The strongest reactions in the immunodiffusion and immunohistochemical methods were found between amyloid deposits of members of a given species and an antibody raised against protein AA from the same species. In contrast to the lack of cross-reactivity in immunodiffusion (except in the mouse-man relationship), extensive cross-reactions were observed immunohistochemically in phylogenetically related species, e.g. between stone marten, pine marten and mink, or between hamster and mouse. However, cross-reactions were also observed in combinations such as man-mouse, man-dog, man-cat, mouse-horse, and dog-cow. In addition, individual antisera showed variations in immunohistochemical reactivity with amyloid deposits of different members of one given species. Moreover, antisera prepared in rabbits reacted more restrictedly than those prepared in sheep, while rabbit antisera against any AA-protein did not react with rabbit amyloid. Finally, the widest degree of cross-reactivity including almost all mammalian species investigated was observed with a chicken antiserum to human amyloid AA protein.

ACKNOWLEDGMENTS

For technical assistance we thank Ms S. Mohr, Ms E. Schmidt-Loock, and Mrs K. B. Linke; for providing amyloid-containing tissue, Privatdozent Dr H.-M. Schneider and Dr S. Störkel, Pathologisches Institut der Universität Mainz, Professor P. Westermark, Institut for Pathology, University of Uppsala, Dr J. Winkelmann, Fa. Nattermann, Köln, and Professor Dr K. Nordstroga, Norges Veterinaerhøyskole, Oslo.

The work was supported by the Deutsche Forschungsgemeinschaft Bonn-Bad Godesberg (Li 247/5–4), by Sonderforschungsbereich 37, H–2 (Munich), by Sonderforschungsbereich 0207, Lp–12 (Munich), and by the Netherlands League Against Rheumatism.

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[*Received for publication, April 2nd,* 1983]