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Tissue distribution of amyloid P component as defined by a monoclonal antibody produced by immunization with human glomerular basement membranes

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Summary

A monoclonal antibody reactive against amyloid P component (NCL-AMP) has been developed following immunization of mice with partially-purified human glomerular basement membranes (GBM) and standard hybridization and cloning techniques. The antibody reactivity was evaluated by enzyme-linked immunosorbent assay (ELISA) and by the indirect immunoperoxidase technique on sections of frozen and fixed human kidney and other tissues. The distribution of amyloid P component in various normal tissues is described and the possible co-localization with the Goodpasture antigen is discussed. In addition, the suitability of the antibody for detection of amyloid deposits in renal amyloidosis is demonstrated and its potential for use in other pathological conditions is considered.

Introduction

Studies on the structure of glomerular basement membranes (GBM) have identified several macromolecules which appear to play important roles in the maintenance of glomerular architecture (Abrahamson, 1986, 1987). These macromolecules, which are variably distributed throughout the GBM and glomerular mesangium, include collagen type IV (Tryggvason *et al.*, 1980) and non-collagenous glycoproteins such as fibronectin (Courtoy *et al.*, 1980); laminin (Sasaki *et al.*, 1987; Sasaki & Yamada, 1987); the glycosaminoglycans, heparan sulphate (Fujiwara *et al.*, 1984), chondroitin sulphate and hyaluronic acid (Lemkin & Farquhar, 1981); entactin (Carlin *et al.*, 1981; Durkin *et al.*, 1988) and amyloid P component (Dyck *et al.*, 1980).

In recent years, considerable attention has been directed towards the identification and location within this complex structure of the antigen responsible for the autoimmune disease Goodpasture's syndrome. It has been demonstrated that the antigen is present in a globular, non-collagenous domain of type IV collagen and that the epitope is sequestered under non-denaturing conditions (Wieslander *et al.*, 1984, 1985; Butkowski *et al.*, 1985, 1987). Further studies have indicated that a new chain ($\alpha 3$) of the collagen IV molecule bears the Goodpasture determinant (Hudson *et al.*, 1989).

Recent studies in our laboratory have focused on the production of monoclonal antibodies using partially-purified human GBM as immunogen. In this paper, we report the production of a monoclonal antibody to amyloid P component (NCL-AMP), which is suitable for immunohistological studies on both frozen and fixed tissue. We present an immunohistological analysis of the distribution of the epitope in normal human kidney and other tissues and comment on its possible co-localization with the Goodpasture antigen. In addition, the use of the antibody for the identification of amyloid deposits in renal amyloidosis is demonstrated and discussed.

Materials and methods

PREPARATION OF HUMAN GBM

Lyophilized GBM was prepared from normal human cadaver kidneys according to the method of Wheeler and Sussman (1981). To prepare a soluble antigen, the lyophilized GBM was

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suspended in 0.1 M Tris-acetate buffer, pH 7.4, containing 0.005 M calcium acetate, to a concentration of 25 mg ml^{-1}. Collagenase was added to 0.7% (w/w) of the GBM and the mixture was incubated at 37°C for 24 h. More collagenase was added, equal to 0.35% (w/w) of the GBM, and incubation was continued for a further 24 h. The mixture was then centrifuged at 3000 **g** for 15 min and the soluble collagenase digest (CD) was stored in aliquots at −20°C.

The protein concentration of CD was determined according to the method of Lowry, as modified by Hartree (1972), with bovine serum albumin as a reference standard.

MONOCLONAL ANTIBODY PRODUCTION

BALB/c mice, aged 6 weeks, were used throughout. All animals were bred and housed in the Comparative Biology Centre of the University of Newcastle upon Tyne and had unrestricted access to food and water.

Mice were immunized with 0.2 ml of CD (40 µg total protein) per injection. A subcutaneous injection of the material in Freund's complete adjuvant was administered initially, followed 15, 28 and 49 days later by injections in Freund's incomplete adjuvant. Blood samples were obtained from tail veins at intervals throughout, and the sera were tested for anti-human GBM antibodies by enzyme-linked immunosorbent assay (ELISA). When high-titre sera were obtained, the antigen was administered intravenously as a solution in sterile 0.01 M phosphate-buffered saline, pH 7.2. Mice were then killed 4 days later by neck dislocation and the spleens removed for the fusion procedure.

Spleen cells were fused with the BALB/c mouse plasmacytoma cell line NS1/1.Ag4.1 (NS1) (Kohler & Milstein, 1975) and monoclonal antibodies derived according to the methods of Hewitt *et al.* (1982) and Routledge *et al.* (1985), as modified by Angus *et al.* (1987). Supernatants were screened initially for anti-GBM activity by ELISA, and positive supernatants were then tested by immunohistochemistry on frozen kidney sections, as detailed below.

NCL-AMP was further characterized by immunoglobulin typing, immunoblotting and reactivity on a range of frozen and fixed tissues, also detailed below.

ELISA FOR MOUSE ANTI-HUMAN GBM ANTIBODIES

The technique was a modification of the method of Wheeler *et al.* (1988). 75 µl volumes were used throughout, and plates were washed three times in 0.01 M phosphate-buffered saline, pH 7.2, containing 0.15% (v/v) Tween 20 (PBST), between each stage. CD at 10 µg ml^{-1} was used as the standard antigen-coating concentration for 3 h at 37°C. Undiluted culture supernatants were added in triplicate and incubated for 2 h at 25°C. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRPO-conjugate) (Dako Ltd), at 1:2000 dilution in PBST, were added and incubated for 1 h at 25°C. Orthophenylenediamine (OPD) was used as substrate, and the reaction was stopped by the addition of 4 M sulphuric acid after 10 min incubation in the dark at room temperature. Positive controls included sera from mice immunized with GBM; negative controls were normal mouse sera and culture medium. Absorbances were read at 492 nm using a Titertek Multiskan MCC ELISA reader (Flow Laboratories) in conjunction with a BBC B microcomputer and a Plateskan program from IQ (Bio) Ltd.

IMMUNOHISTOCHEMISTRY

Fixatives, buffers and reagents

Fixatives (70% ethanol, formalin, formal sublimate, acetone, glutaraldehyde, paraformaldehyde and Bouin's solution), buffered washes and staining solutions were prepared according to standard procedures (Bancroft & Stevens, 1982).

Human tissues

All specimens were obtained from the Department of Histopathology, Royal Victoria Infirmary, Newcastle upon Tyne. Specimens of normal human kidney, skin, liver, lung and spleen were obtained at autopsy and were either snap-frozen in carbon dioxide or fixed in the variety of fixatives stated above and routinely processed to paraffin wax. In addition, frozen sections of routine renal biopsies were obtained from three patients with renal amyloidosis.

Indirect immunoperoxidase technique

Fixed tissue. Sections, 2–4 µm, on poly-l-lysine-coated slides were treated by standard de-waxing and rehydration procedures. To inactivate endogenous peroxidase, sections were immersed in 0.5% hydrogen peroxide in methanol for 10 min at room temperature. After rinsing in tap water, the sections were treated with 0.1% trypsin in Tris-buffered saline (TBS) containing 0.1% calcium chloride (pH 7.8) for between 5 and 15 min. After a further rinse in tap water, the sections were incubated with 20% normal rabbit serum (NRS) in TBS to block non-specific binding of the secondary antibody. Excess NRS was removed and replaced with primary antibody [hybridoma supernatants (neat to 1-in-100 in NRS) or mouse monoclonal antibody to human collagen IV (Dako Ltd) (1-in-60 in NRS)]. Sections were then incubated overnight at 4°C in humidified incubation trays. Following two 5-min rinses in TBS, sections were overlaid with secondary antibody, rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRPO-conjugate) (Dako Ltd), at 1-in-40 in NRS, and incubated for 1 h at room temperature. Sections were again rinsed twice in TBS, 5-min per rinse. The peroxidase reaction was developed with 3,3′-diaminobenzidine (1 mg ml^{-1}) plus imidazole (0.68 mg ml^{-1}) in TBS, pH 7.6, containing 0.02% hydrogen peroxide, for 2–3 min. Sections were counterstained with Mayer's haematoxylin (Boenisch, 1989).

Negative control sections were included; they were incubated with NRS in place of the primary or secondary antibodies or both. All sections were coded and assessed semiquantitatively.

Frozen tissue. Frozen tissue sections were air-dried for 3 min, fixed in acetone for 10 min and again air-dried. They were then treated with NRS for 20 min to block non-specific binding of the secondary antibody. The remainder of the procedure was as detailed above for fixed tissue sections.

Peroxidase anti-peroxidase technique

Frozen tissue sections were air-dried for 3 min, fixed in acetone for 10 min and again air-dried. Sections were then incubated with normal swine serum (NSS), 20% in TBS, to minimize non-specific binding of the secondary antibody (swine anti-rabbit immunoglobulins) (Dako Ltd). Sections were subsequently flooded with primary antibody, rabbit anti-human serum amyloid P component (Dako Ltd), diluted 1-in-20 in NSS, and

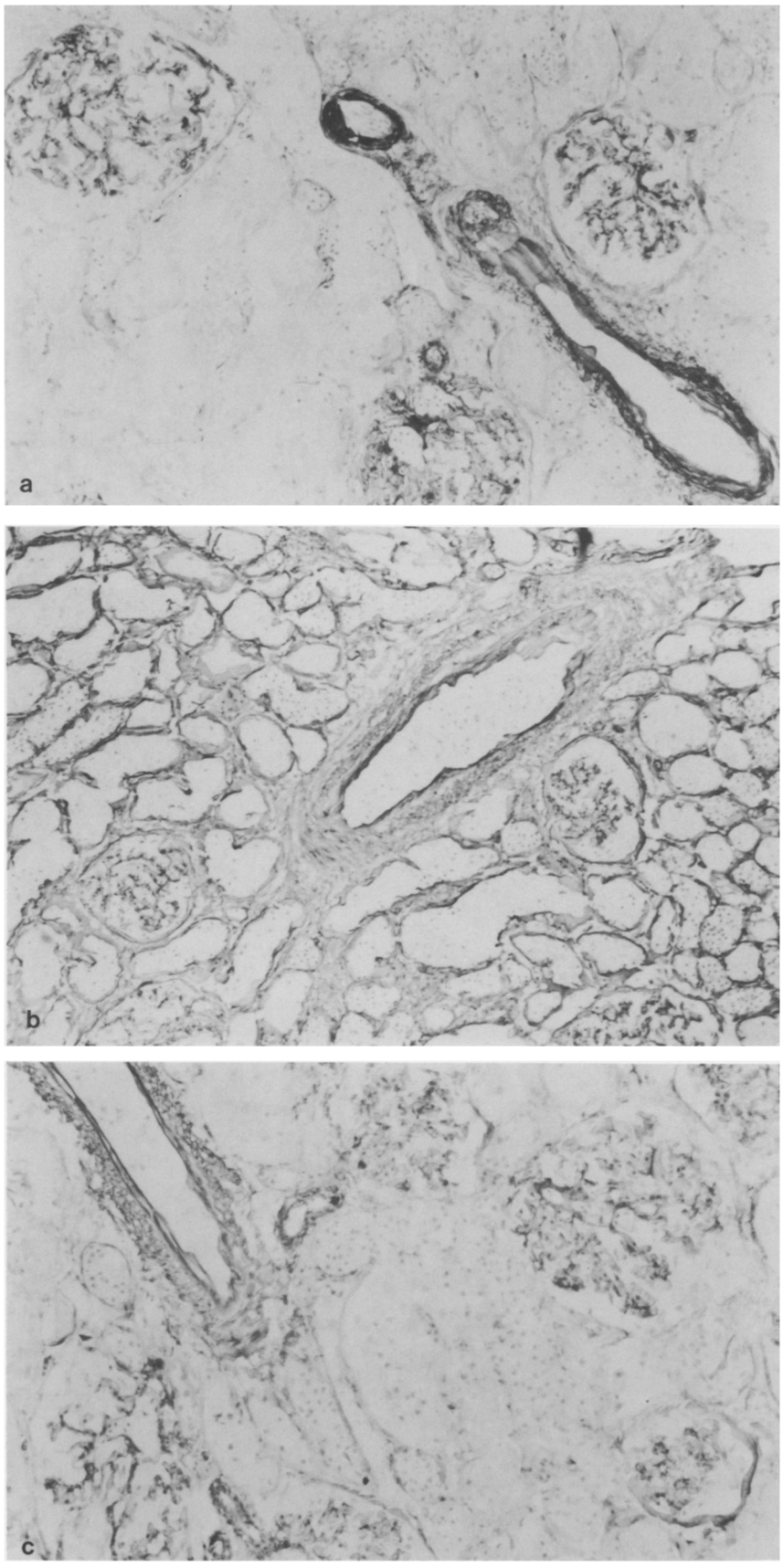


Fig. 1. Indirect immunoperoxidase technique showing reactivity in frozen human kidney of (a) NCL-AMP and (b) mouse anti-human collagen IV. (c) Peroxidase anti-peroxidase technique showing reactivity of rabbit anti-human serum amyloid P component in frozen human kidney.

Table 1. Reactivity of NCL-AMP in frozen kidney: comparison with reactivity of other antibodies and staining patterns obtained by histological techniques

	Antibodies			*Histology*	
Features	*NCL-AMP*	*Collagen IV*	*Amyloid P*	*Reticulin*	*Elastin*
GBM	+ +	+ +	+ +	+	−
Bowman's capsule	Partial	All	Partial	All	−
TBM	Very weak	+ +	Very weak	+ +	−
Blood vessels	+ +	+ +	+ +	+ +	+ +

GBM = glomerular basement membrane; TBM = tubular basement membrane.

incubated for 30 min at room temperature. Following two 5-min washes in TBS, sections were incubated with secondary antibody at a dilution of 1-in-20 in NSS. The sections were again rinsed twice in TBS (5 min per rinse), covered with rabbit peroxidase anti-peroxidase (Dako Ltd), at 1-in-50 dilution in NSS, for 30 min at room temperature and then rinsed twice in TBS. The peroxidase reaction and counterstaining were as for the indirect immunoperoxidase technique.

Negative control sections were included; they were incubated with NSS in place of primary or secondary antibodies or both. All sections were coded and assessed semiquantitatively.

IMMUNOGLOBULIN CLASS DETERMINATION

Immunoglobulin typing was assessed with a mouse monoclonal typing kit (ICN Immunobiologicals), which uses Ouchterlony immunodiffusion.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE) AND IMMUNOBLOTTING

CD was separated by SDS-PAGE and the separated components transferred to nitrocellulose as described by Towbin *et al.* (1979). After washing, 3 ml of NCL-AMP supernatant was overlayed on the nitrocellulose filter for 3 h at 37°C, with gentle shaking. The filter was washed to remove unbound material and then reacted with HRPO-conjugate for 2 h at 37°C. Bands of reactivity were detected by incubating the filter in 4-chloro-1-naphthol and hydrogen peroxide for 30 min in the dark at room temperature.

Standards of known molecular weight were used to assist with the interpretation of the pattern obtained.

HISTOLOGICAL METHODS

Standard histological methods for elastin (Miller, 1971), reticulin (Gordon & Sweet, 1936) and amyloid (Highman, 1946) were performed on frozen tissues, by the Department of Histopathology, Royal Victoria Infirmary. All sections were coded and assessed semiquantitatively.

Results

PRODUCTION OF NCL-AMP

The fusion from which NCL-AMP was developed resulted in approximately 50 hybridoma colonies. Repeated screening of supernatants from these colonies by ELISA indicated that only one of these (NCL-AMP) was producing immunoglobulins strongly reactive with the soluble human GBM antigen preparation. In addition, NCL-AMP showed specific reactivity when used to immunostain sections of frozen kidney.

CHARACTERIZATION OF NCL-AMP

Immunoglobulin class and immunoblotting

The Ouchterlony immunodiffusion technique demonstrated that NCL-AMP was of the class IgG1. Immunoblotting studies against CD of GBM indicated that NCL-AMP recognized three polypeptide bands: a strong band at 28.8 kDa and two weaker closely-associated bands of molecular weight 50.1 and 49.8 kDa.

Reactivity of NCL-AMP on frozen human tissues

With the indirect immunoperoxidase technique, NCL-AMP showed reactivity against a variety of frozen normal human tissues.

In human kidney, NCL-AMP showed a linear staining pattern along the GBM, but only partial staining of

Table 2. Reactivity of NCL-AMP in frozen skin: comparison with reactivity of other antibodies and staining patterns obtained by histological techniques

	Antibodies			*Histology*	
Features	*NCL-AMP*	*Collagen IV*	*Amyloid P*	*Reticulin*	*Elastin*
BM/DEJ	−	+ +	−	+ +	−
BM/SG	+ +	+	+ +	−	+
Elastic fibres	+ +	−	+ +	−	+ +
Reticular fibres	−	−	−	+ +	−
Collagen fibres	−	−	−	+ +	+ +

BM = basement membrane; DEJ = dermoepidermal junction; SG = sweat gland.

Table 3. Reactivity of NCL-AMP in frozen liver: comparison with reactivity of other antibodies and staining patterns obtained by histological techniques

	Antibodies			*Histology*	
Features	*NCL-AMP*	*Collagen IV*	*Amyloid P*	*Reticulin*	*Elastin*
Portal tract	+ +	+ +	+ +	+ +	+ +
Endothelial BM of central vein	+ +	+ +	+ +	+ +	+ +
Sinusoidal BM	−	+ +	−	−	−
Capsule	+ +	+ +	+ +	+ +	+ +

BM = basement membrane.

Table 4. Reactivity of NCL-AMP in frozen spleen: comparison with reactivity of other antibodies and staining patterns obtained by histological techniques

	Antibodies			*Histology*	
Features	*NCL-AMP*	*Collagen IV*	*Amyloid P*	*Reticulin*	*Elastin*
Trabeculae	+ +	+ +	+ +	+ +	+ +
Trabecular arteries and veins	+ +	+ +	+ +	+ +	+ +
Venous sinuses	−	+ +	−	−	−
Capsule	+ +	+ +	+ +	+ +	+ +

Bowman's capsule, adjacent to the vascular pole. There was no significant staining in the mesangium. Occasionally, there was a very weak staining of the tubular basement membranes (TBM) but this was not easily distinguishable from background levels. However, NCL-AMP reacted strongly with the elastic layer in the 'peri-connective tissue' of blood vessels (Fig. 1a).

This staining pattern in kidney was then compared with that obtained using a monoclonal antibody to human collagen IV (Fig. 1b) and standard histological methods for reticulin and elastin. These results are summarized in Table 1. Anti-collagen IV and the silver stain for reticulin gave similar results: a strong staining of the GBM, TBM, all of Bowman's capsule and renal blood vessels. The elastin stain also strongly highlighted renal blood vessels but, in contrast, did not react with any basement membranes.

Taken together, the above results suggested that NCL-AMP reactivity was not to collagen IV but to a component present in both GBM and elastic fibres. As amyloid P is one such component, frozen kidney sections were immunostained with a polyclonal antibody to human serum amyloid P component, using the peroxidase anti-peroxidase technique. Although a higher non-specific background staining was evident, the overall pattern of staining in the GBM and blood vessels was the same as that obtained with NCL-AMP (Fig. 1c and Table 1), indicating its reactivity against amyloid P component.

The immunohistochemical and histological stains, as used on human kidney, were then applied to other frozen human tissues, namely, skin, liver, spleen and lung. The results obtained are shown in Tables 2 to 5 and, in all cases, the staining pattern observed with NCL-AMP (Fig. 2) again mirrored that seen with the polyclonal antibody to amyloid P component.

Incubation of NCL-AMP with either CD or pure serum amyloid P component (kindly provided by Dr M. R. Barer, Department of Microbiology), prior to immunohistochemical application, abolished all subsequent staining, confirming that NCL-AMP is specific for an epitope of amyloid P component. This was confirmed by Dr J. Raynes (Institute of Hygiene and Tropical Medicine), using an ELISA with serum amyloid P component as coating antigen.

Table 5. Reactivity of NCL-AMP in frozen lung: comparison of reactivity with other antibodies and staining patterns obtained by histological techniques

	Antibodies			*Histology*	
Features	*NCL-AMP*	*Collagen IV*	*Amyloid P*	*Reticulin*	*Elastin*
Alveolar BM	+	+ +	+	−	−
Alveolar capillary walls	+ +	+ +	+ +	+ +	+ +
Pleural surface	+ +	−	+ +	+ +	+ +

BM = basement membrane.

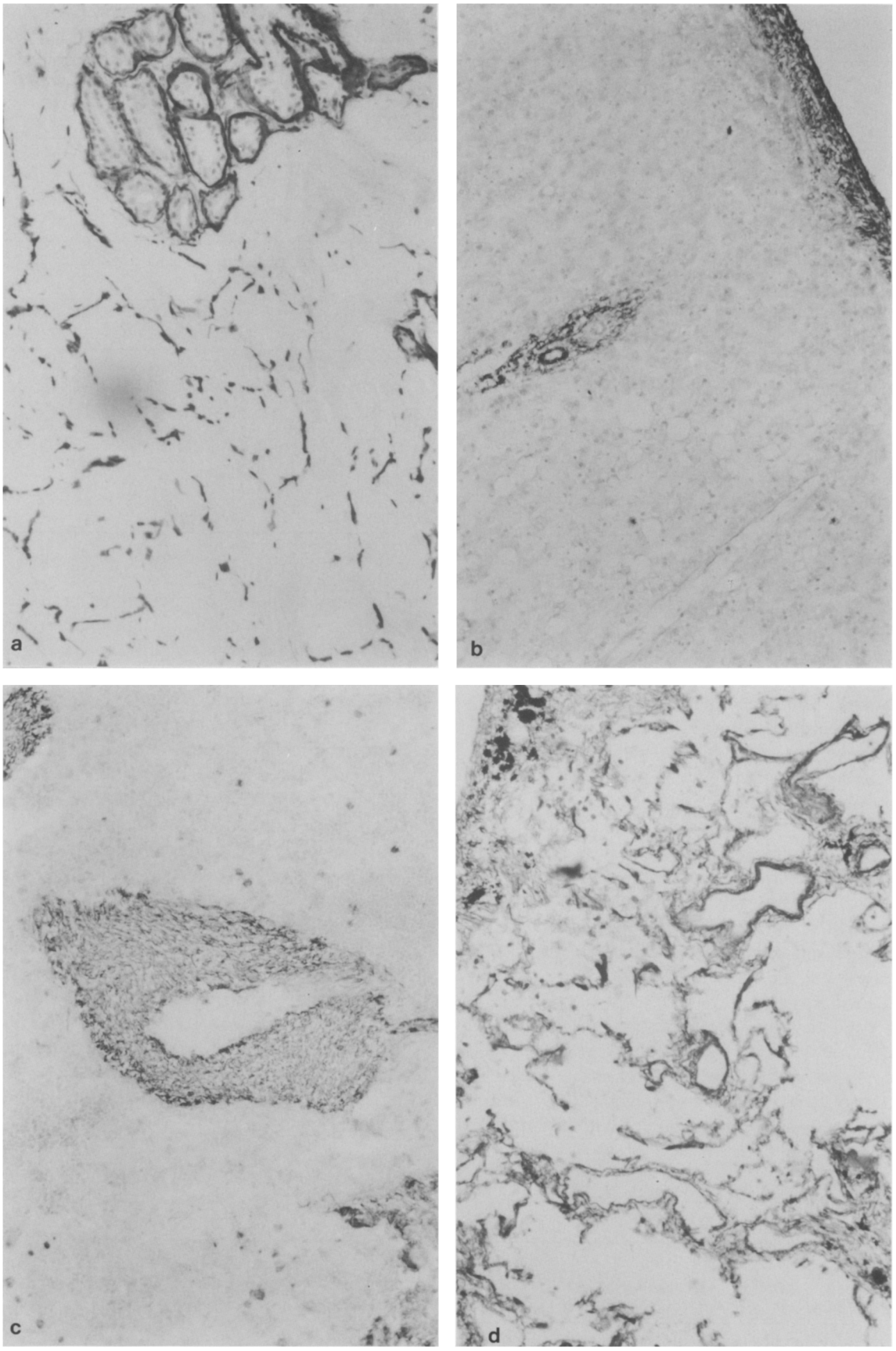
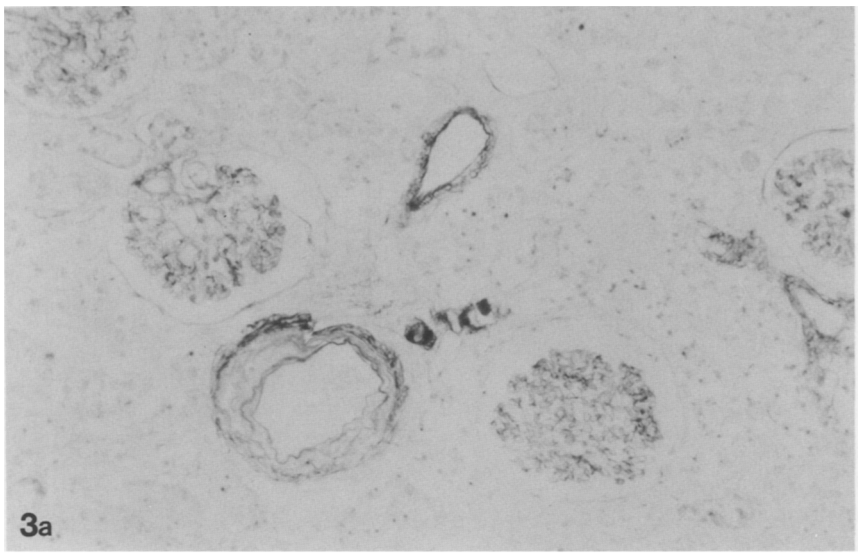
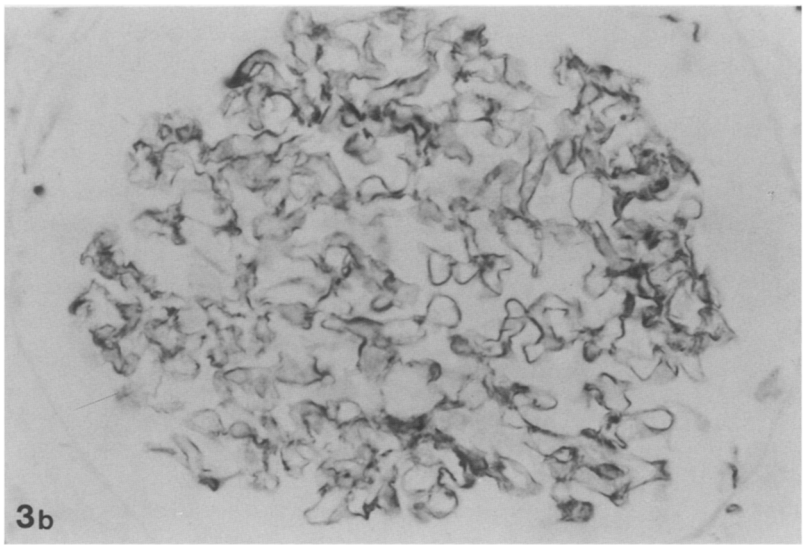


Fig. 2. Indirect immunoperoxidase technique showing reactivity of NCL-AMP in frozen human tissues: (a) skin, (b) liver, (c) spleen, (d) lung.





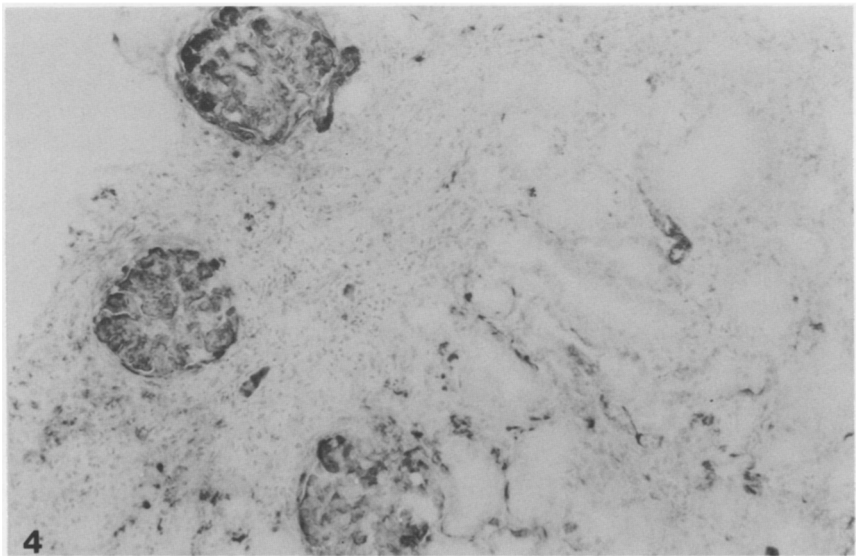


Fig. 3. Indirect immunoperoxidase technique showing reactivity of NCL-AMP in acetone-fixed human kidney: (a) low magnification, (b) high magnification of an individual glomerulus.

Fig. 4. Indirect immunoperoxidase technique showing reactivity of NCL-AMP in renal amyloidosis.

REACTIVITY OF NCL-AMP ON FIXED HUMAN KIDNEY

Reactivity of NCL-AMP was subsequently assessed by the indirect immunoperoxidase technique on human kidney fixed by a range of fixatives, as detailed in 'Materials and methods'. Reactivity was evident following fixation in acetone or 70% ethanol and trypsinization for 5, 10 or 15 min (Fig. 3a, b). The distribution of staining was identical to that observed in frozen kidney. Reactivity was not observed with other fixatives, even after prolonged trypsinization.

APPLICATIONS FOR NCL-AMP

Frozen sections of kidney from three cases of renal amyloidosis were immunostained with NCL-AMP by the indirect immunoperoxidase technique. Positive staining was seen in amyloid deposits in glomeruli, in the peritubular area and in the vascular walls, as shown in Fig. 4. All immunostaining was again abolished by prior incubation of NCL-AMP with either CD or pure serum amyloid P component.

Similar results were obtained with the polyclonal antibody to serum amyloid P component, using the PAP technique.

This distribution of immunostaining with both antibodies corresponded to that of congophilic amyloid deposits, as demonstrated with the Highman congo red stain.

Discussion

Using a soluble form of human GBM as the immunogen, we have produced a murine monoclonal antibody (IgG1) to amyloid P component (NCL-AMP), previously identified as a normal covalently-linked matrix protein of GBM (Dyck *et al.*, 1980). Reactivity of the antibody was primarily evaluated on the basis of similarities and differences of staining of the structural elements within tissue sections, using other monoclonal and polyclonal antibodies and standard histological techniques. Final confirmation of reactivity was by ELISA, with serum amyloid P component as coating antigen.

Immunoblot analysis of collagenase-digested GBM with NCL-AMP revealed three bands of molecular weight 28.8, 49.8 and 50.1 kDa. These results are similar to those reported by Dyck *et al.* (1980), who suggested that amyloid P subunits are associated with different peptide chain fragments, possibly derived from collagen or other matrix proteins, following digestion with bacterial collagenase.

The production of NCL-AMP has allowed us to assess the distribution of amyloid P component in various tissues (kidney, skin, liver, spleen and lung). In all cases, amyloid P component was found to be closely associated with elastic fibres, confirming the results of Mera and Davies (1987). In addition, in the kidney, amyloid P component appears to be confined mainly to the GBM, unlike collagen IV, which is extensively distributed in all renal basement membranes. This confirms the results of Dyck *et al.* (1980), who found that glomerular and other vascular basement membranes contained an antigen that was immunochemically indistinguishable from serum amyloid P component.

The distribution of amyloid P component demonstrated by NCL-AMP closely follows that of the Goodpasture antigen (Pusey *et al.*, 1987) in showing a linear pattern in the GBM, involving Bowman's capsule, and in the alveolar basement membranes (ABM), with the difference that amyloid P component was demonstrated only very weakly in distal tubules. The significance of this observation is uncertain. While the Goodpasture antigen has been localized to the globular, non-collagenous domain of the $\alpha 3$ chain in basement membrane collagen IV, there is no information about the localization of basement membrane amyloid P component, even though it may represent some 10% of the collagenase-solubilized GBM (Dyck *et al.*, 1980). The distribution of Goodpasture antigen has been quantified recently by inhibition ELISA (Weber *et al.*, 1990), being at the greatest concentration in the GBM (100%), TBM (66%), ABM (48%) and intestinal membranes (33%). This widespread distribution does not coincide with the histological lesions of Goodpasture's syndrome, which are confined to the GBM and ABM. One may speculate, therefore, that the co-distribution of amyloid P component with Goodpasture antigen in the GBM and ABM could determine localization of lesions. Dyck *et al.* (1980) have indicated that serum amyloid P component can bind both modified C3 and microbial polysaccharides. A similar reactivity of amyloid P component in GBM and ABM could potentially direct immunological attack to these sites. Further, indirect evidence for the co-distribution of Goodpasture antigen and amyloid P component in the GBM is seen in Alport's syndrome, as, in this hereditary nephritis, both antigens are uniformly absent (Melvin *et al.*, 1986).

The absence of reactivity of NCL-AMP on tissue treated with aldehyde-containing fixatives, even after trypsinization, would suggest destruction or masking of the relevant amyloid P epitope by such cross-linking, non-coagulant fixatives. In contrast, the positive reactivity of NCL-AMP on tissue fixed in acetone or ethanol reflects the mild denaturing effects of these fixatives, which allow antibody penetration and generally do not block immunoreactive determinants. The requirement for trypsinization after fixation in acetone or ethanol is somewhat unusual but appears to reflect the need for partial proteolysis of this epitope after such fixation.

Although amyloid P component is a normal constituent of many tissues, the unequivocal demonstration of amyloid deposits with NCL-AMP suggests that this antibody may have diagnostic potential, not only in cases of renal amyloidosis but also in other situations where amyloid may play a pathological role, for example in Alzheimer's disease and in other types of cerebral amyloidosis (Coria *et al.*, 1988).

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