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Detection of both isotypes of complement C4, C4A and C4B, in normal human glomeruli

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Detection of both isotypes of complement C4, C4A and C4B, in normal human glomeruli. Monoclonal antibodies reactive against the complement C4A and C4B isotypic components were used in an immunoperoxidase technique for the histological study of normal human renal tissue. Prominent staining with both antibodies was seen in the mesangial areas of all normal kidney sections investigated. Occasional staining of arteriolar walls of the same tissues, however, was also observed. In contrast, no mesangial staining was seen using monoclonal antibodies reactive against other 'early' complement components, such as C1q and C3. Specificity of the glomerular staining with the anti-C4 reagents was demonstrated in two patients possessing only the C4A serum component but lacking genetically the C4B locus products. As would be predicted, glomerular staining with the anti-C4A reagent, but not anti-C4B, was clearly demonstrable. It is concluded that both isotypes of complement C4 are present in normal human glomeruli and thus might be operative for normal mesangial function.

The HLA region is assumed to control immune responsiveness in man, and particular alleles of several HLA encoded genes are useful markers of disease [1, 2]. Several studies have already examined possible associations between HLA class I and class II antigens and glomerulonephritis [3]. In addition, alleles of the HLA-linked complement genes (class III products), C4, C2 and factor B, are also used as genetic markers of disease [4]. The fourth component of human complement, C4, which is controlled by two highly polymorphic genes, C4A and C4B [5, 6], has been particularly useful in this respect. It has been shown recently that a rare variant of the C4B locus, termed C4B2.9, is significant as an immunogenetic marker in patients with glomerulonephritis [7]. This disease association with C4 raises the question, therefore, whether the genetically determined, structural variants of C4 might be important in the pathogenesis of diseases such as glomerulonephritis. Detection of the early complement components C1q, C4 and C3, usually by immunofluorescence, is interpreted as being due to deposition of immune complexes [8], either of preformed, circulating immune complexes, or by in situ complex formation [9]. Since such staining is not observed in normal kidney, detection of these complement components is usually indicative of glomerulonephritis.

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In this study, we have used two monoclonal antibodies reactive against C4 isotypic components (anti-C4A and anti-C4B) in an immunoperoxidase staining technique on normal human kidney, and demonstrate in the mesangium the presence of the two isotypes, C4A and C4B, of complement C4.

Methods

Normal kidney tissue

Kidney tissue was obtained from ten patients undergoing nephrectomy due to renal carcinoma. Macroscopically normal tissue was kept in tissue culture medium, immediately snap–frozen and stored at −80°C until preparation of cryostat sections. Only histologically normal, tumor–free sections, as judged by light microscopy, were used for immunoperoxidase staining. In addition, percutaneous renal biopsies were obtained from two patients with isolated proteinuria. One biopsy was taken from a patient with acute pulmonary embolism immediately after non-successful resuscitation. One sample was obtained at autopsy within eight hours postmortem from a patient with myocardial infarction. All samples appeared normal on microscopic examination and did not demonstrate immune complex deposition.

Immunoperoxidase staining

Immunoperoxidase staining was performed with modifications as described in detail previously [10]. In brief, 5 μm cryostat sections were used. The sections were fixed in acetone for 10 minutes followed by a short wash in phosphate–buffered saline (PBS) at pH 7.4. They were then incubated with the appropriately diluted, primary monoclonal antibodies for 60 minutes. After incubation the sections were thoroughly washed in PBS, and then reacted with peroxidase–conjugated rabbit anti-mouse immunoglobulins (Dakopatts, Copenhagen, Denmark) for 60 minutes. After final washing in PBS, the sections were stained with 3-amino-9-ethylcarbazole (Sigma Chemical Corp., St. Louis, USA) and H_2O_2 (Merck, Darmstadt, W. Germany) for 20 minutes in PBS. Counter–staining was done with hemalaun, and the sections were mounted with glycerine–gelatine (Merck) for microscopic evaluation.

When polyvalent antisera (two rabbit antisera, one goat antiserum) were used as primary antibodies, the sections were then reacted with peroxidase–conjugated swine anti-rabbit

immunoglobulins (Dakopatts) and peroxidase–conjugated rabbit anti-goat (Sigma), respectively.

Monoclonal antibodies

Mouse monoclonal antibody (G2B12) reactive against C4B locus products has been described and characterized in detail elsewhere [11]. It reacts with a determinant on the α-chain of all C4B variants.

Mouse monoclonal antibody (GIC4) against C4A locus products reacts with a common determinant on the α-chain of all C4A variants.

Mouse monoclonal antibody against C4d is commercially available (Cytotech, San Diego, USA).

Mouse monoclonal antibody against C1q has been characterized in detail elsewhere [12].

Monoclonal antibodies against various C3-epitopes have been described elsewhere [13].

Monoclonal antibodies against human IgG and IgM are commercially available (Dianova GmbH, Hamburg, W. Germany).

Polyvalent anti-C4 antisera

Polyvalent rabbit immunoglobulins anti-human C4 were purchased from two commercial sources (Dakopatts and Calbiochem–Behring Corp., La Jolla, California, USA). These antisera were used in immunoperoxidase staining of tissue sections, in immunoblotting of serum samples and in inhibition studies.

Polyvalent goat–immunoglobulins anti-human C4 (Atlantic antibodies, Scarborough, USA) were used in immunoperoxidase staining and in immunoblotting.

Inhibition of monoclonal antibody binding by polyvalent antisera

Possible blocking of monoclonal antibody binding to the α-chain of C4 by two polyvalent rabbit–anti-C4 antisera was investigated. Kidney sections were incubated with polyvalent anti-C4 immunoglobulins in various dilutions for 60 minutes prior to immunoperoxidase staining with monoclonal anti-C4 antibodies. Monoclonal anti-C4 antibodies were also reacted with kidney sections in the presence of excess polyvalent rabbit–anti-C4 antibodies. Immunoperoxidase staining was performed as described.

In addition, human C4 was immunoprecipitated from serum with either polyvalent goat or rabbit anti-C4, subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose filters. The blots were then incubated with excess polyvalent rabbit–anti-C4 prior to staining with monoclonal anti-C4 antibodies and peroxidase–conjugated rabbit anti-mouse immunoglobulins (Dakopatts).

Immunoblotting of C4 was performed as described [11]. C4 electrophoretic variants were determined in neuraminidase–treated EDTA-plasma by immunofixation electrophoresis in agarose gels [5].

Results

Two murine monoclonal anti-human C4 antibodies, one reactive only with the C4A isotype (GIC4) and the other (G2B12) reactive only with C4B, were used in an immunoperoxidase technique for the histological study of normal renal tissue.

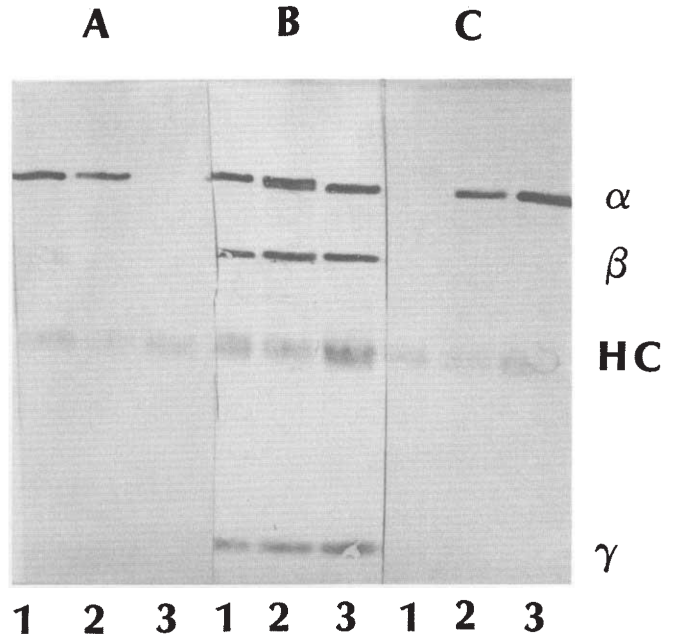

Fig. 1. *Reactivity of monoclonal anti-C4 antibodies determined by SDS-PAGE analysis of immunoprecipitated C4 from individuals of different C4 phenotype followed by immunoblotting.* The C4 phenotypes of the samples are: track 1, C4A only (C4A3); 2, C4A + C4B (C4A3, B1); and 3: C4B only (C4B1). In **A**, the blot was developed with anti-C4A (GIC4) with no staining in track 3 (that is, individual lacking C4A). In **B**, the blot was developed with a polyclonal antiserum to C4 (goat) revealing staining of the three chains of C4 (α, β and γ). In track 2, two molecular weight forms of the C4α-chain are apparent corresponding to C4A-α (96.000 mol wt) and C4B-α (94.000 mol wt). In *C*, the blot was developed with anti-C4B (G2B12) with no staining in track 1 (that is, individual lacking C4B). Note that only the α-chain of C4 is stained with the monoclonal reagents. Abbreviation HC is IgG heavy chain.

Reactivity of monoclonal anti-C4A and anti-C4B antibodies

The reactivity pattern of these monoclonal anti-C4 reagents is shown in Figure 1. Here the reactivity of the monoclonal anti-C4 reagents is compared to a polyclonal anti-C4 antiserum (goat) using SDS-PAGE analysis of immunoprecipitated C4 from serum of individuals of different C4 phenotype, followed by immunoblotting. It can be seen that in contrast to the polyclonal reagent, both monoclonal antibodies were specific for an epitope expressed on the C4-α chain, whereas the polyclonal reagent was reactive with all three subunit chains (α, β, and γ). Furthermore, the anti-C4A monoclonal antibody (GIC4) was reactive only with the α-chain of C4A (96.000 mol wt) and not C4B, whereas the anti-C4B reagent (G2B12) was reactive only with the α-chain of C4B (94.000 mol wt) and not C4A. The anti-C4A reagent was reactive with all C4A gene products identified so far [14] and the anti-C4B reagent was reactive with all well–defined C4B gene products [14].

Immunoperoxidase staining of kidney tissue

Figure 2 illustrates representative staining of tumor–free kidney tissue from one nephrectomy out of ten. Similar results

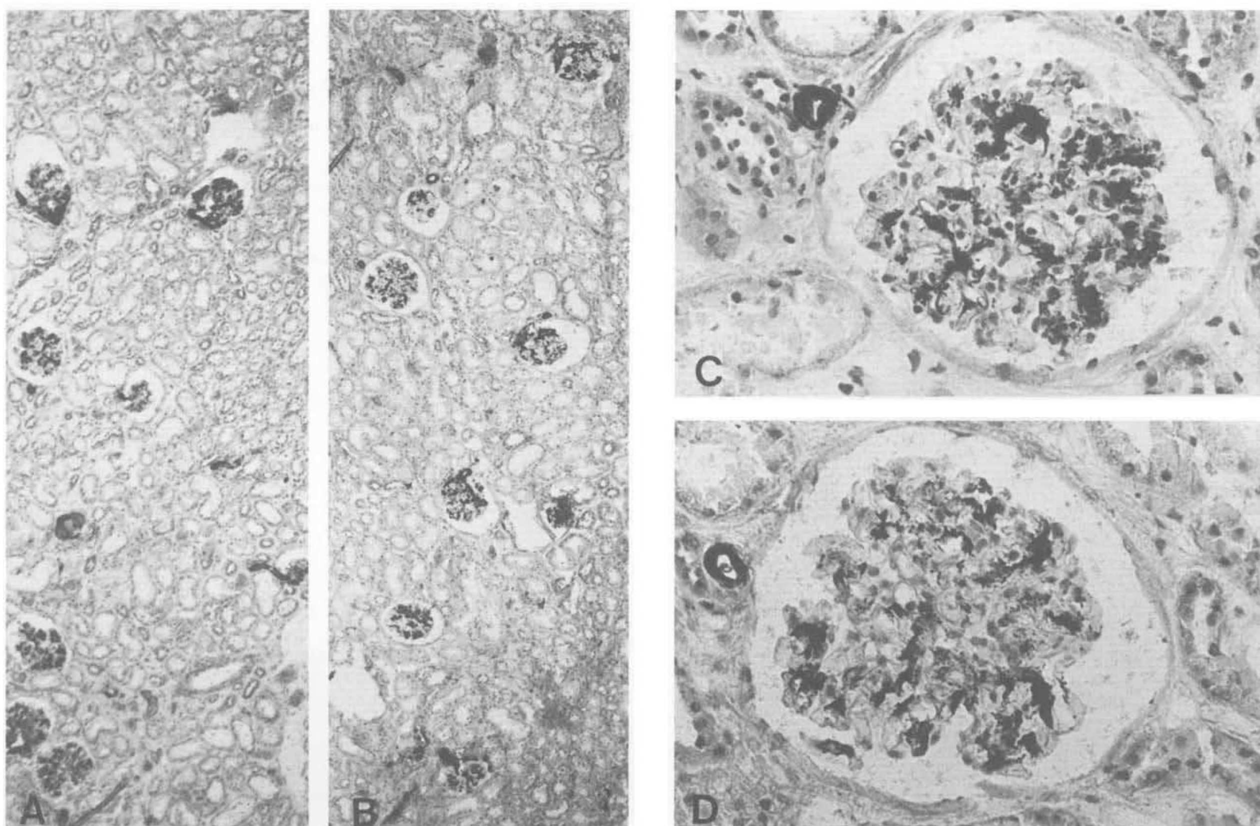

Fig. 2. *Staining of normal human kidney with monoclonal antibodies against C4A and C4B complement components.* **A** and **B**. Survey of renal cortex with intensive staining of all glomeruli with the anti-C4A reagent (**A**) and the anti-C4B reagent (**B**) (×30). **C** and **D**. Higher magnification (×210) of one glomerulus with staining of the mesangial area with anti-C4A (**C**) and with anti-C4B (**D**). Note also staining of arteriolar vessel wall.

were obtained with all other normal kidney specimens investigated.

The staining pattern observed with the monoclonal antibody against C4A was identical to that obtained with the antibody against C4B, with intense staining in the mesangial areas of all glomeruli. The staining appears to be cellular and was equally distributed in the different mesangial regions. In addition, there was staining of arteriolar vessel walls at the vascular pole of the glomerulus. Occasionally, positive vascular extensions beyond the glomerulus could be detected. It should also be noted that interstitial arterioles showed distinct reactivity, although weaker than that of the mesangial areas. Other vascular elements as well as constituents of the tubular system were, however, consistently negative. Identical staining could be seen in three experiments with the monoclonal anti-C4d antibody.

From these observations using the anti-C4 reagents, it was important to determine if other 'early' complement components, such as C1q and C3, could be similarly detected in normal human kidney. Thus, a monoclonal antibody against C1q and three against different C3 epitopes were also used in the immunoperoxidase staining of normal kidney. None of these antibodies showed any reactivity with normal kidney sections. Two different polyvalent rabbit antisera and one goat antiserum against C4 were similarly negative with normal human kidney tissue. Neither reacted with monoclonal antibodies against human IgG and IgM with normal kidney (data not shown).

To further examine the specificity of the tissue staining with the monoclonal anti-C4A and anti-C4B antibodies, we investigated cryostat sections obtained from kidney biopsies of two patients. One patient, a 24-year–old woman, had the clinical and laboratory findings of Systemic Lupus Erythematosus. The other biopsy was taken from a 54-year–old man with insulin–dependent diabetes mellitus and nephrotic syndrome; histological examination showed diabetic glomerulosclerosis. Complement C4 allotyping using immunofixation electrophoresis revealed that both patients possessed the C4A serum component (C4A3) but lacked the C4B products in their serum. As would be predicted on the basis of the C4 allotyping results, in both patients only the anti-C4A antibody showed staining of the mesangial area (Fig. 3A, SLE patient), whereas the anti-C4B reagent was completely negative (Fig. 3B).

Inhibition of monoclonal antibody binding by polyvalent antisera

In three experiments, polyvalent rabbit antisera failed to block the binding of monoclonal anti-C4A and anti-C4B reagents, both in immunoperoxidase staining of kidney sections and in immunoblotting of serum samples.

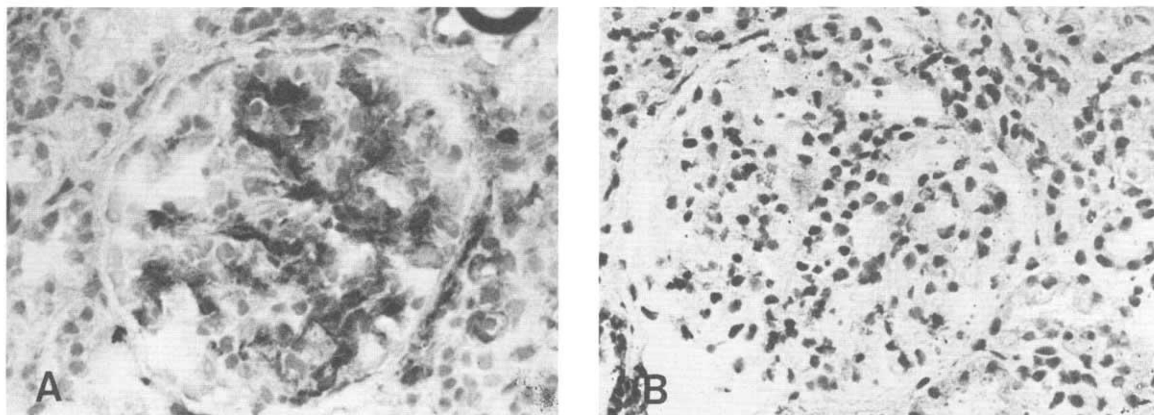

Fig. 3. *Biopsy specimen of a SLE-patient possessing only the C4A serum component but lacking genetically the C4B locus products.* Note staining of mesangial area with antibody against C4A (**A**). Absence of staining with antibody against C4B (**B**) (×210).

Discussion

The main finding in this study is the demonstration of both isotypes of complement C4, C4A and C4B, in normal human glomeruli. This observation is further substantiated by positive staining obtained with a monoclonal antibody reactive against C4d. The notable absence of other 'early' complement components, such as C1q and C3 as well as IgG and IgM, suggests the exclusive mesangial presence of C4, independent from immune complex deposition.

The function of the glomerular mesangium seems to be essential for homeostasis in renal physiology [15]. Mesangial cells appear to be heterogeneous, and different functions are displayed by these cellular subpopulations [16]. A considerable portion of mesangial cells is believed to be of macrophage/monocyte lineage. Experimental data point to their important role in the removal or degradation of macromolecules and immune complexes [17, 18]. It is uncertain, however, whether the mechanisms of this clearance can be attributed solely to phagocytosis by these cells, or whether other cellular functions are involved. Membrane determinants such as Fc-receptors [19] and receptors for C3b [20] have been observed in human glomeruli and are thought to facilitate the uptake of debris material. In addition, several 'immunological' cell surface markers, including HLA class II antigens and Thy 1 antigen, have been identified on rat and human glomerular cells [17, 21, 22]. Their biological role within the glomerulus, however, remains far from clear.

From the present study, the detection of HLA-class III components, that is, the isotypes of complement C4 in normal human glomeruli, raises questions regarding the origin and function of C4 in the kidney. The staining pattern obtained with our monoclonal antibodies indicates the expression and/or production of C4A and C4B by mesangial cells. It is apparent that not all mesangial cells express C4, since the staining is not homogeneous throughout the mesangium. It remains to be determined whether the cell population that expresses HLA-class III antigens is identical with the population carrying HLA-class II antigens.

The mesangial presence of C4 should be discussed in the context of possible pathogenic mechanisms in autoimmune diseases. Thus, mesangial cells may utilize the many biological properties of complement C4, including its participation in antigen processing and in the induction of a humoral immune response [23]. Of particular importance would be its well documented ability to bind to immune complexes [24] with subsequent solubilization via activation of C3 [25]. It is certainly of interest that individuals with either a total or partial deficiency of serum C4 are prone to the development of Lupus Erythematosus [26, 27]. Further evidence for a direct role of C4 in disease pathogenesis has been suggested from the observation that drugs, such as hydralazine, that induce a lupus–like syndrome, inhibit C4 in vitro [28]. Partial C4 deficiency has also been reported in increased frequency in IgA nephropathy, Henoch Schönlein Purpura and benign recurrent hematuria [29, 30].

Immunohistological staining of kidney biopsies from two patients, one with SLE, the other with diabetic glomerulosclerosis, and both lacking the C4B serum component, demonstrated the specificity of staining with the monoclonal anti-C4 reagents.

Glomerular staining was only seen using the anti-C4A antibody not, as would have been predicted, with the anti-C4B reagent. Thus, it seems unlikely that there is some inadvertent cross–reactivity of the antibodies with other mesangial matrix components, such as fibronectin.

The assumption that the isotypes of complement C4 would be expressed and function independently from each other is of interest in terms of the reported difference in biological activity between the two isotypes. It was recently shown that C4B is about fourfold more active in immune hemolysis than C4A [31]. Similarly, the structural polymorphism of C4 might result in functional heterogeneity. For example, the rare variant of C4, C4B2.9, which is an immunogenetic marker in primary glomerulonephritis [7], may be deleterious in terms of maintaining regular mesangial function. The functional activity of the C4B2.9 variant compared to other C4 variants should, therefore, be determined.

From these immunohistological studies it cannot be determined whether C4 is passively adsorbed on mesangial cells or whether it is actively produced by these cells. To solve this problem, studies on isolated or cultured mesangial cells are required. Passive adhesion of C4 in the form of cytophilic

immune complexes seems unlikely, however, since C1q and C3 were not detected in any of the tissue sections. Preliminary results from patients with primary glomerulonephritis indicate that the staining pattern of immune complexes is clearly different from that of normal kidney.

The staining of some arteriolar vessel walls with the monoclonal anti-C4 reagents also deserves further studies. Noteworthy is the close relationship of mesangial cells and smooth muscle cells in vessel walls which has recently been recognized [32]. Studies with fetal kidney cells are required to determine a possible common origin of these cell types.

The reason why C4 was not demonstrable in normal kidney using polyvalent antisera is not entirely clear. Since polyvalent rabbit antisera failed to block monoclonal antibody binding in immunoperoxidase staining and in immunoblotting, it is likely that these reagents detect different or not accessible epitopes.

In conclusion, the studies indicate that C4 may be a normal mesangial constituent of human kidney. The local presence and putative function of both isotypes would provide a new clue to our understanding of the direct involvement of immunogenetic factors in the pathogenesis of renal autoimmune diseases.

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