Activation of the Alternative Pathway of Complement by Monoclonal λ Light Chains in Membranoproliferative Glomerulonephritis

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Summary

Immunopathological evidence suggests that activation of the alternative pathway of complement (AP) is involved in membranoproliferative glomerulonephritis (MPGN) and in immunoglobulin A nephropathy. In this report we describe an AP dysfunction-associated factor that was isolated from the serum and urine of a patient with hypocomplementemic MPGN. Extensive glomerular deposits of C3, properdin, and of the terminal complement components were observed in the kidney of the patient. In her serum the AP hemolytic activity was virtually absent. When mixed with fresh normal serum, the patient's serum induced a 96% C3 conversion during a 30-min incubation at +37°C. This activity was found to be due to a circulating factor that by immunochemical characterization proved to be a 46-kD monoclonal immunoglobulin λ light (L) chain dimer (λ_L). Purified λ_L, but not control λ or κ L chains from patients with L chain disease, activated the AP in a dose- and ionic strength–dependent manner. Functionally, λ_L was differentiated from C3 nephritic factor (an autoantibody against the AP C3 convertase, C3bBb) by its inability to bind to and stabilize the C3bBb enzyme. Instead, λ_L was observed to interact directly with the AP control factor H. Thus, λ_L represents a novel type of immunoglobulin-related AP-activating factor with the capacity to initiate alternative complement pathway activation in the fluid phase.

The alternative pathway of complement (AP)[1] is considered to play a crucial role in protection against infectious diseases (1, 2). It promotes phagocytosis and can cause cytolysis of microbes, virus-infected cells, and some tumor cells. Target structures for the AP are usually recognized in a discrimination process, which involves downregulation of an interaction between target-associated C3b molecules and control factor H (3–5).

An association between AP activation/dysfunction and human glomerulonephritis has been observed in several studies (6–8). The involvement of the AP in kidney disease has been judged from immunofluorescence analysis of kidney biopsy specimens (deposition of complement components in glomeruli) and from changes in total serum complement activity or in levels of individual components. In one subset of glomerulonephritis, membranoproliferative glomerulonephritis (MPGN) type II, characterized by intramembranous dense deposits in glomerular basement membranes, the AP dysfunction has been shown to be due to an abnormal autoantibody in patient sera. This IgG antibody, C3 nephritic factor (C3 Nef), reacts with and stabilizes the newly assembled AP C3 convertase, C3bBb (9–11). Stabilization of C3bBb by C3 Nef causes its increased resistance to spontaneous or factor H–mediated decay dissociation resulting in prolonged survival of the enzyme (12, 13) and in an accelerated turnover of C3, which is the substrate for the C3bBb enzyme.

In this report we describe a novel type of factor, an Ig λ L chain dimer (λ_L), with a capacity to initiate activation of the alternative pathway in the fluid phase. This factor was isolated from the serum and urine of a patient with hypocomplementemic MPGN. Studies of λ_L have revealed a previously unrecognized interaction between an Ig fragment and the alternative pathway activation system.

[1] *Abbreviations used in this paper:* AP, alternative pathway of complement; C3 Nef, C3 nephritic factor; EM, electron microscopy; IFL, immunofluorescence; IFX, immunofixation; LM, light microscopy; MPGN, membranoproliferative glomerulonephritis; NHS, normal human serum; P, properdin.

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Materials and Methods

Case History and Patient Samples. The patient (AL), a 57-yr-old Caucasian woman with no history of serious illness, presented with weight loss (10 kg/mo), anemia (hemoglobin level, 95 g/liter), and renal insufficiency, as indicated by increased serum creatinine (up to 1,200 μmol/liter, normal range 70–106 μmol/liter) and urea (up to 40 mmol/liter, normal range, 2.3–6.5 mmol/liter) levels. 1 mo before, she had suffered from upper respiratory and gastrointestinal tract infections of unknown cause. Analysis of the urine showed microscopic hematuria and proteinuria (4.2 g/liter). Proteins in the urine contained originally 0.78 g/liter of free Ig λ L chains (and <0.05 g/liter of κ L chains), as studied by a polyethylene glycol–enhanced radial immunodiffusion procedure using mAbs specific for free human λ L chains (The Binding Site, Birmingham, UK). Immunofixation (IFX) analysis (see below) of the urine showed a monoclonal band reacting with polyclonal anti-λ antiserum (Behringwerke, AG, Marburg, FRG). Protein electrophoresis of the patient serum revealed a weak extra band in the β1 region. It could be immunofixed with antisera to λ L chains, but not with antisera to κ L chains nor with antisera to γ, μ, α, δ, or ε H chains. No evidence for multiple myeloma was obtained from histological analysis of bone marrow biopsy specimens or from x-ray studies during a follow-up period of 2 yr.

Both serum and EDTA (0.01 M) plasma samples were taken from the patient on six occasions with an interval of 1.3 yr between the first and last samples. Samples were stored in aliquots at −70°C before analysis. Plasmapheresis plasma and urine samples were also obtained and kept at −20°C before fractionation procedures. A kidney sample was obtained by a percutaneous needle biopsy under ultrasound guidance. Normal human sera (NHS) were obtained from healthy laboratory personnel. Serum from a patient with poststreptococcal glomerulonephritis (PSGN) and sera from patients with L chain myelomas were obtained from the district of the University Central Hospital of Helsinki and stored at −20°C.

Microscopical Studies. The renal biopsy specimen was processed for light (LM), immunofluorescence (IFL), and electron microscopic (EM) study according to standard methods. For IFL microscopy, pieces of the biopsy were frozen in isopentane cooled with liquid nitrogen. Cryostat sections (5 μm) were fixed in 3.5% paraformaldehyde in PBS, pH 7.4, for 10 min and washed three times for 5 min with PBS. The bound complement components or Igs were detected by incubating the sections with fluorochrome-conjugated (rabbit or goat) antisera to C1q, C4, C3c, IgG, IgA, or IgM (Behringwerke AG). Other complement components or Ig L chains were detected by first incubating the sections with unlabeled polyclonal (rabbit or goat) antibodies to C3d, P, H, C5, C6, C7, C8, C9 (Quidel Corp., San Diego, CA), κ, or λ L chains (Behringwerke AG), or with a mouse anti-iC3b neospecific mAb (Quidel Corp.), followed by incubation with a second, fluorochrome-conjugated antibody (goat anti–rabbit IgG or rabbit anti–goat IgG; Behringwerke AG). Sections were analyzed by using a standard microscope equipped with epi-illumination and interference filters for fluorescein and rhodamine fluorescence (excitation filter BP 455-490, dichroic mirror FT510, emission filter LP 520; Osram HBO 50W high pressure mercury lamp; Carl Zeiss, Inc., Thornwood, NY). For photography, Kodak Tri-X film was used.

Purified Complement Proteins and Preparation of Complement Intermediates. Complement components C3, H, B, and D were purified according to procedures modified from earlier studies (5, 14, 15). The complement components used in the fluorimetric assay were purified at the Department of Biochemistry, University of Texas Health Center (Tyler, TX) (5, 14, 16). Native, functionally active C3 was separated from inactive C3 by FPLC on a mono S column (Pharmacia, Uppsala, Sweden). Properdin was isolated as described (17, 18). Sheep erythrocytes bearing C3b (EC3b) and the active AP C3 convertase (EC3bBb) were generated at +30°C using C3, B, D, and Ni^{2+} in gelatin (0.1%) veronal buffered saline (VBS) buffer, pH 7.4 (Ni-GVB; 4, 19).

Hemolysis Tests for Complement Activity and Methods to Detect Cleavage of C3, C4, C5, and Factor B. Alternative pathway hemolytic activity was tested by hemolysis of guinea pig or rabbit erythrocytes, and classical pathway hemolytic activity by hemolysis of antibody-sensitized sheep red cells using both tube hemolysis and hemolysis-in-gel assays (20). Hemolytic tests for C3 Nef activity were according to Arnaout et al. (21). Antigenic levels of the individual AP components were determined by single radial immunodiffusion.

C3 conversion to C3b/iC3b was analyzed by an IFX electrophoresis method as described before (22). The anaphylatoxins C3a desArg (23), C4a desArg (24), and C5a desArg (23) were quantitated by an RIA (Amersham International, Amersham, UK). The AP-activating capacity of whole sera, C3 conversion, and generation of the anaphylatoxins were analyzed from mixtures of NHS and patient serum after a 30-min incubation at +37°C. C3 activation was analyzed both in PBS, pH 7.4, and in MgEGTA-PBS (0.005 M $MgCl_2$, 0.01 M EGTA in PBS). 0.01 M EDTA (chelator of both Mg^{2+} and Ca^{2+}) was used as a control to block both pathways of complement activation. To test for AP activation by purified Ig L chains, these were added at varying concentrations, dissolved in VBS, pH 7.4, into fresh NHS, and C3 cleavage was studied by C3a RIA.

Gradient (8–20%) SDS-PAGE was performed according to Laemmli (25). For reduction of the samples, 2-ME was used. The molecular mass markers (Bio-Rad Laboratories, Richmond, CA) used were hen egg white lysozyme (14.4 kD), soybean trypsin inhibitor (21.5 kD), bovine carbonic anhydrase (31 kD), hen egg white OVA (42.7 kD), BSA (66.2 kD), and rabbit muscle phosphorylase b (97.4 kD). As a radioactive protein standard a ^{14}C-methylated protein mixture (Amersham International) was used: lysozyme (14.3 kD), carbonic anhydrase (30 kD), OVA (46 kD), BSA (69 kD), phosphorylase b (92.5 kD), and myosin (200 kD). Electrotransfer and immunoblotting were according to Towbin et al. (26). Analyses of factor B and C5 conversion were done using nitrocellulose replicas from agarose and SDS-PAGE gels. The nitrocellulose sheets were stained with peroxidase-conjugated specific antisera against factor B (rabbit polyclonal), Bb fragment (mouse monoclonal), or C5 (rabbit polyclonal; all antisera were from Quidel Corp.).

Fractionation of Patient Serum. Initially, 150 μl of serum from patient AL was applied to a Superose 6 gel filtration column (Pharmacia) equilibrated with PBS, pH 7.4. Protein concentration was followed by measuring the absorbance at 277 nm. To determine the complement-activating capacity of the chromatography fractions, equal volumes of each fraction and NHS were mixed and incubated for 30 min at +37°C, and the resultant C3 cleavage was analyzed by both the C3 conversion IFX and C3a generation assays. Fractions were also analyzed by nondenaturing protein electrophoresis. IgG, IgA, and IgM were quantitated by radial immunodiffusion against polyclonal antisera (Behringwerke AG). The presence and monoclonality of free λ L chains in chromatography fractions were assessed by Ouchterlony and IFX analysis, respectively. Free λ L chains were quantitated by a combined densitometer scanning of the IFX gel and single radial immunodiffusion using free λ L

chain chain-specific antiserum. Reactivity with the anti-Fd antiserum (Behringwerke AG) was tested by Ouchterlony double immunodiffusion.

Purification of the Complement-activating Factor. 2 ml of the patient serum was precipitated with 40% ammonium sulphate in PBS, pH 7.4. The supernatant was dialyzed overnight against 10 mM diaminopropane, 0.01 M HCl, pH 8.3, containing 0.025% NaN_3, and applied to a mono Q anion exchange column (Pharmacia). The column was eluted with a NaCl gradient (0–0.5 M) at a flow rate of 600 μl/min. Fractions (1.8 ml) were tested for the presence of monoclonal and polyclonal Igs by Ouchterlony double diffusion using both anti-κ and anti-λ antisera, the former precipitating only the polyclonal Igs. The ability of the fractions to activate complement was tested after dialysis against PBS.

The complement-activating factor was purified from patient urine using a SI17 column (an anion exchange column; Pharmacia). 300 ml of urine was dialyzed against 10 mM phosphate buffer, pH 7.4, and concentrated before application to the column. The column was run at a flow rate of 1 ml/min and proteins were eluted with a NaCl gradient collecting 3-ml fractions. Protein concentration was followed by absorbance at 277 nm, and λ L chains were detected by Ouchterlony double diffusion.

Monoclonal λ L chains from patient plasmapheresis plasma and control λ (λ_V, $\lambda_{\hat{A}}$) and κ (κ_W) L chains from the sera and urines of patients with L chain myelomas were isolated as described above for serum. Proteins were radiolabeled with ^{125}I using the Iodobeads technique (Pierce Chemical Co., Rockford, IL) to specific activities of 0.5–1.0 μCi/mg.

Cation Exchange Chromatography of Patient Serum. 500 μl of serum was applied to a CM-TSK column (Bio-Rad Laboratories) in 10 mM phosphate buffer, pH 6.2, (flow rate: 600 μl/min). Fractions were tested for the presence of λ L chains by double immunodiffusion and IFX analysis. Positive fractions were pooled and rechromatographed on the same column with a NaCl gradient (conductivity from 8.5 to 44 millisiemens [mS]) in 25 mM phosphate buffer, pH 5.5. Superose 6 gel filtration was used as the final step to separate λ L chains from albumin.

Binding of λ_L to E_S-C3b and E_S-C3bBb. The purified and radiolabeled λ_L or control λ L chains (λ_V, $\lambda_{\hat{A}}$) were added at varying concentrations (up to 60 μg/ml) to suspensions of E_S, E_S-C3b, or E_S-C3bBb (6×10^8 cells/ml) in a final volume of 100 μl gelatin veronal buffer (GVB) and incubated with stirring for 30 min at 30°C. Cell-bound radioactivity was determined after centrifugation of the mixtures through 20% sucrose. The percentage of L chain binding was obtained by counting the radioactivity in pellets and supernatants. For hemagglutination experiments, polyclonal antisera against λ L chains, C3c, and factor B (Behringwerke AG) were used. Residual C3 convertase activity was assayed by incubating the cells with normal rat serum diluted 1:15 in GVB-EDTA at 37°C for 60 min. Cell lysis was determined as hemoglobin release (absorbance at 412 nm).

Fluorescence Quenching Assay for C3bBb Stabilizing Activity. Cleavage of C3 to C3b by the enzyme C3bBb was assayed fluorimetrically essentially as described earlier (27, 28). C3bBb enzyme was preformed by mixing and incubating (2 min at 22°C) 22.5 μg C3b (generated by trypsin), 96 μg factor B, 2.5 μl 50 mM $MgCl_2$, and 0.8 μg factor D in a final volume of 60 μl of VBS. Formation of the enzyme was stopped by adding 60 μl of ice-cold VBE (VBS with 0.04 M EDTA). 40 μl of the enzyme was added immediately after its formation to cuvettes containing native C3 (193 μg/ml), the fluorescent probe ANS (8-anilino-1-naphtalene sulphonate, 40 μM), and either properdin (P; 40 μg/ml), λ_L (71 μg/ml), or a corresponding amount of HBS (20 mM Hepes, 140 mM NaCl) buffer, pH 7.4. C3 cleavage was followed by a spectrofluorometer (8000-C; SLM Instruments, Urbana, IL) with excitation and emission wavelengths of 386 and 472 nm, respectively.

Binding of λ_L to Factor H. Flexible microtiter plate wells (Linbro; Flow Laboratories, Inc., Hamden, CT) were coated (overnight at +4°C) with factor H, C3b, or BSA (all at 10 μg/ml) in VBS. After blocking of unbound binding sites with 0.1% gelatin in VBS-Tween 20, 0.1% (VBS-T), the plates were thoroughly washed with VBS-T, and dilutions of radiolabeled λ_L L chains (1.5×10^6 cpm/μg) were added to microtiter plate wells in duplicate and incubated for 1 h at room temperature. After removal of the radiolabeled proteins, the plates were washed four times with VBS-T and individual wells cut out and counted in a gamma counter.

Sucrose Density Gradient Analysis. ^{125}I-labeled λ_L (final concentration, 0.45 μg/ml) was incubated (30 min at +37°C) with purified factor H (0.75 mg/ml), BSA (2 mg/ml), or with NHS (diluted 1:2), and layered on top of 10–30% sucrose gradients in PBS. Gradient tubes were centrifuged for 18 h at 40,000 rpm using a SW50 rotor in an ultracentrifuge (L-70; Beckman Instruments Inc., Palo Alto, CA). Fractions (140 μl) were collected from the bottom of the tubes and counted for radioactivity. Hb, BSA, IgG, and IgM were used as migration markers.

Results

Glomerular Lesion: LM, EM, and IFL Microscopic Findings. The kidney biopsy specimen of the patient was studied by routine histological and immunohistological methods. By LM, all (~15) glomerular capillary tufts in the specimen were moderately condensed due to an increase in the mesangial matrix, thickening of the capillary walls, and slight proliferation of mesangial and endothelial cells. In silver staining, the peripheral capillary glomerular basement membrane (GBM) appeared "duplicated" (Fig. 1 *A*), suggesting mesangial interposition or intramembranous deposits. The tubules were atrophied, and the interstitium was heavily fibrotic and infiltrated by round cells. One small artery showed destruction of the vessel wall with infiltration of round cells, i.e., vasculitis.

Two glomeruli were studied by EM. In both, ~50% of the capillary loops contained intramembranous-subendothelial deposits of moderate electron density (Fig. 1 *B*). Similar deposits were present in the mesangial matrix. Some capillaries showed circumferential mesangial interposition. No deposits were detected in Bowman's capsule or tubular basement membranes.

By IFL, intensive coarse granular deposits of C3 were detected along the capillary walls and in the mesangial regions (Fig. 2), when stained with antisera (anti-C3c), that recognize C3, C3b, iC3b, and C3c. There was similar, but weaker, staining when anti-iC3b-neoantigen antiserum was used. Anti-C3dg antisera showed an extensive linear staining along capillary basement membranes. Properdin (Fig. 3 *A*) and the membrane attack complex components C5 (Fig. 3 *B*), C7, and C9 were present as deposits similar to those of C3, although the staining reactions with the respective antisera were slightly weaker. The alternative pathway control factor H was also found deposited in the kidney glomeruli (Fig. 3 *C*). Factor H depositions were less intense than those of C3 and they appeared in a diffuse linear fashion along the basement mem-

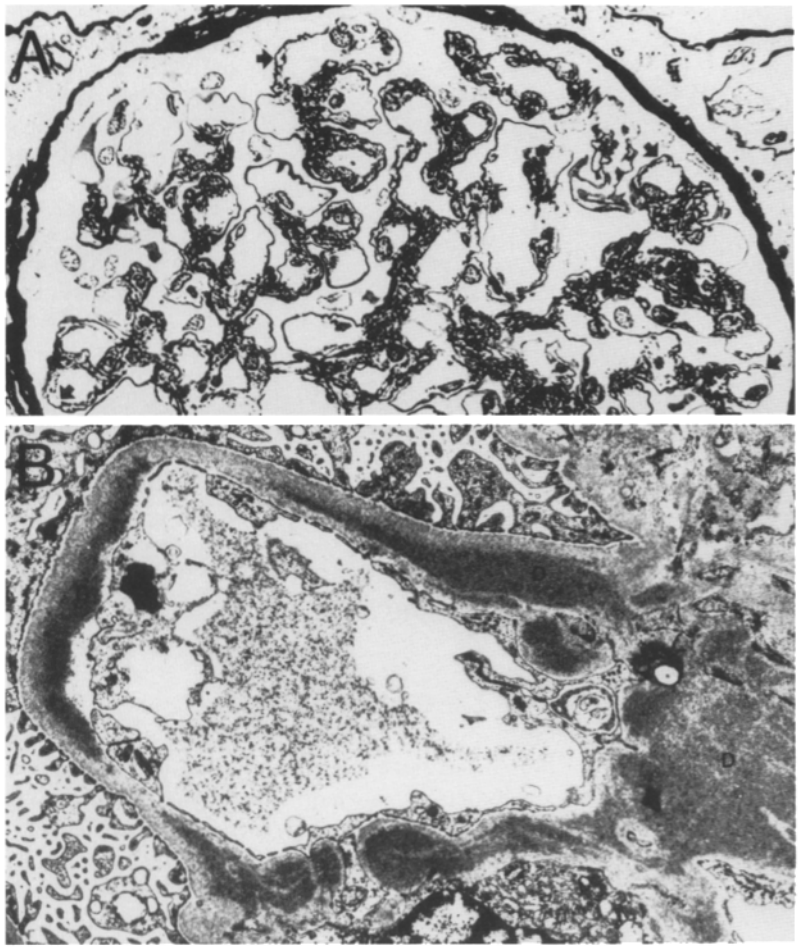

Figure 1. (*A*) LM (silver stain) and (*B*) EM appearance of the patient kidney biopsy specimen. An increase in the mesangial matrix and and thickening of the capillary walls are evident. At several sites (*A*), the glomerular basement membrane appears duplicated apparently because of dense intramembranous-subendothelial deposits (*B*) in the capillary loops. The pattern corresponds to an atypical form of MPGN with features of both types I (subendothelial deposits) and II (dense intramembranous deposits).

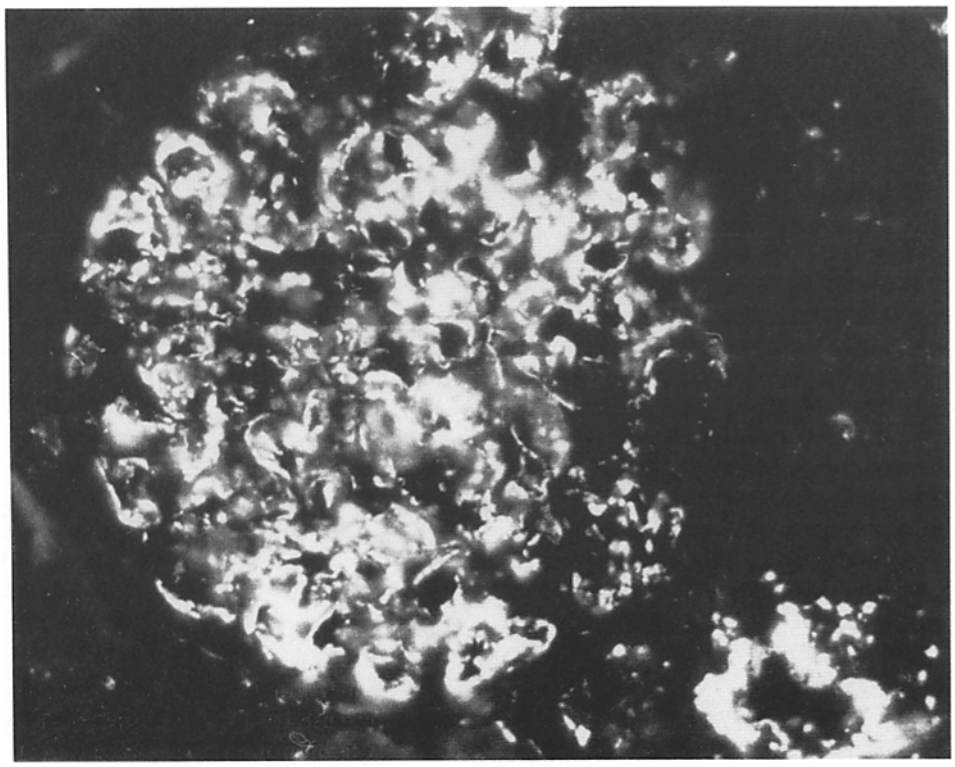

Figure 2. IFL microscopy demonstration of intense C3 deposits in a glomerulus and in an artery wall. Unfixed cryostat section of the biopsy specimen was labeled with a FITC-labeled antiserum against C3c (×650).

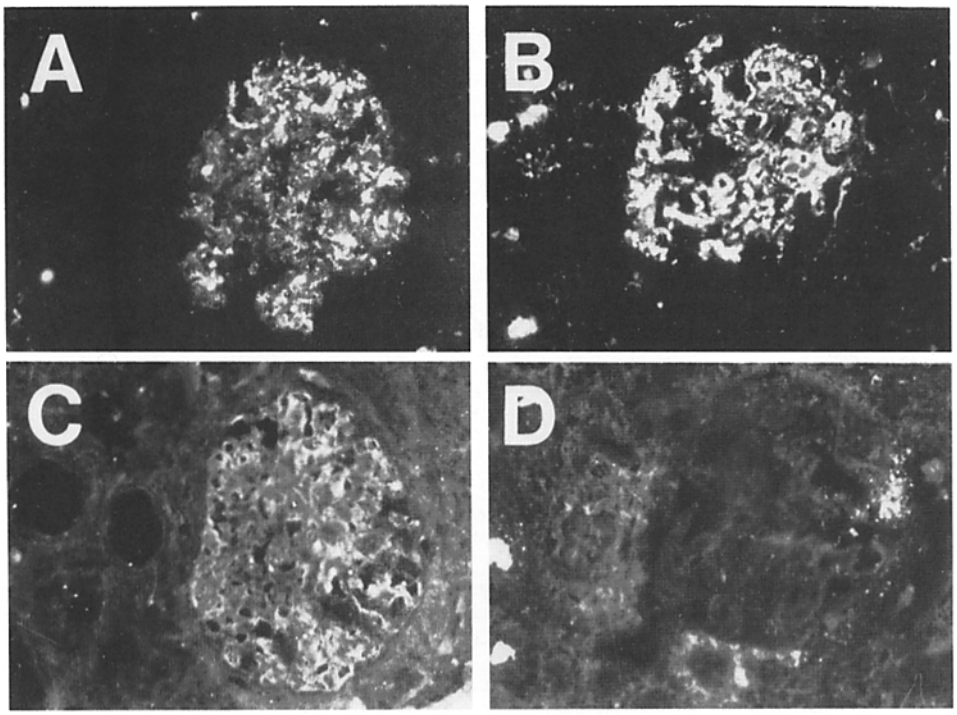

Figure 3. Immunofluorescence microscopy detection of (*A*) properdin, (*B*) C5, and (*C*) factor H in glomeruli. (*D*) Staining for IgG does not show glomerular deposits (×200).

branes and not as coarse granular deposits. No deposits of C1q, C4, or Igs (with H chain–specific antisera) were detected. Ig λ, but not κ, L chains were occasionally seen deposited in damaged glomeruli and as tubular casts (Fig. 4). In some glomeruli, λ L chains appeared faintly along the basement membranes similarly as factor H.

Complement Activity and Igs in Serum. The tube hemolysis titration and the hemolysis-in-gel (hemolysis of guinea pig or rabbit erythrocytes) analysis of the fresh patient serum showed a markedly decreased AP hemolytic C activity (<20%, compared with a pool of normal control sera), but the classical pathway hemolytic activity was at the normal level (75 IU/ml; cut off level, 60 IU/ml). Levels of the individual alternative pathway C components, as determined by single radial immunodiffusion, were low, but detectable: C3, 0.07 g/liter (normal range, 0.6–1.4 g/liter); factor B, 0.06 g/liter (0.1–0.4 g/liter). Factor H (122%), factor I (138%), and C4 (0.39 g/liter, 0.2–0.6 g/liter) were present at normal antigenic levels in the patient serum. Antisera against properdin gave only a weak precipitin band in an Ouchterlony double diffusion test. The IgG, IgA, and IgM concentrations in serum were within normal range (not shown). No rheumatoid factors or cryoglobulins were detected, and IFL tests for antiglomerular and antitubular basement membrane antibodies in serum were negative.

Detection of a Complement-activating Factor in Serum. The low levels of C3 and B, but not of C4, in patient serum suggested the presence of C3 nephritic factor. A nearly total (96%) C3 conversion was obtained after mixing and incubating (30 min at +37°C) the patient serum with fresh NHS (Fig. 5).

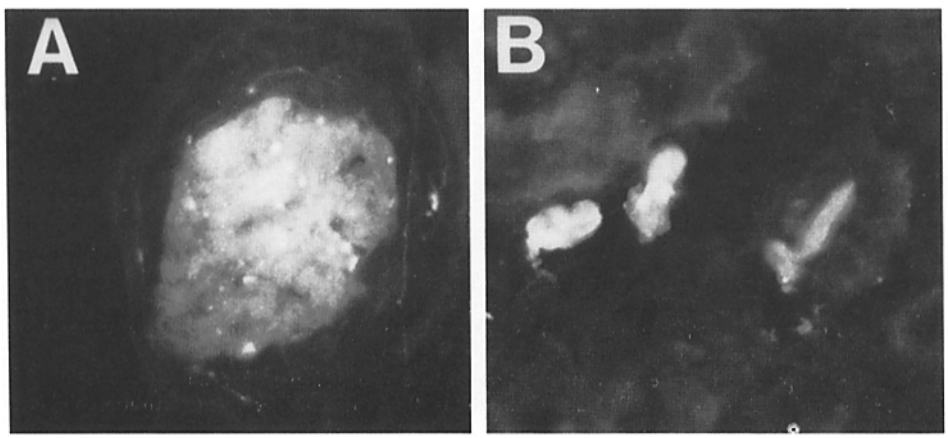

Figure 4. IFL detection of λ L chains in patient kidney. λ L chains can be found (*A*) deposited in damaged glomeruli or (*B*) as tubular casts (×400).

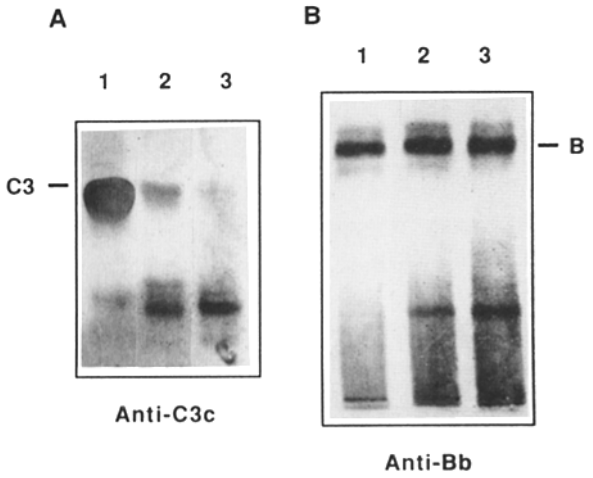

Figure 5. Demonstration of (*A*) C3 and (*B*) factor B cleavage in NHS by patient serum. NHS alone (lane *1*), NHS with zymosan, 1 mg/ml (lane *2*), or a mixture of patient serum and NHS (1:1) (lane *3*) were incubated for 30 min at +37°C and subjected to electrophoresis in agarose. C3 cleavage was studied by IFX with a polyclonal anti-C3c antiserum. Activation of factor B was analyzed by immunoblotting using a monoclonal anti-Bb antiserum on a nitrocellulose replica of an agarose gel. Note the somewhat different C3 cleavage patterns induced by zymosan and patient serum: a virtually complete and homogenous cleavage of C3 is induced by the patient serum.

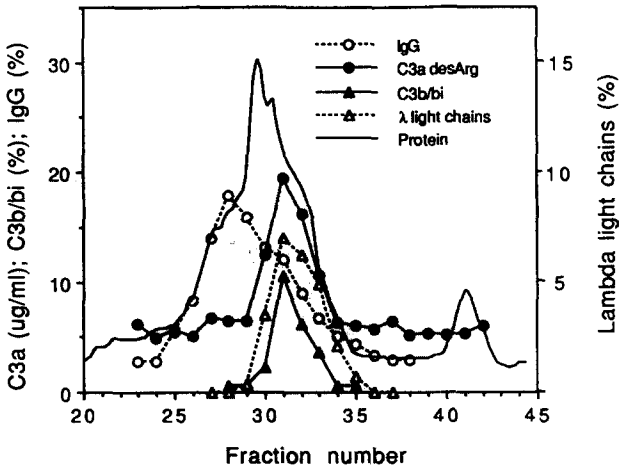

Figure 6. Comigration of C3 cleaving activity with monoclonal λ L chains in gel filtration analysis of patient serum. 150 μl of patient serum was fractionated on a Superose 6 column. Protein concentration was followed by absorbance at 277 nm. Complement-activating capacity of the chromatography fractions was tested by the C3 conversion IFX and C3a generation assays. The presence and monoclonality of free λ L chains in chromatography fractions were assessed by the immunodiffusion and IFX analysis, respectively. Levels of IgG and free λ L chains were quantitated by single radial immunodiffusion using monospecific antisera and by quantitative densitometer scanning of IFX gels. Results are expressed as percent of total input (1.8 mg of IgG and 105 μg of λ L chains).

This C3 conversion was inhibited with 0.01 M EDTA but not with 0.01 M MgEGTA (5 mM $MgCl_2$; Table 1). Factor B (Fig. 5) and C5 (C5a desArg formation) were also cleaved, but not C4 (C4a desArg), which shows that activation occurred through the alternative pathway (Table 1). NHS was depleted of alternative pathway hemolytic activity when mixed with patient serum at a ratio of 1:1 and incubated for 30 min at +37°C. It is thus apparent that the patient serum contains some factor(s) capable of activating the AP in serum. The activity persisted in the patient serum during a follow-up period of 1.3 yr, as shown by analyzing five samples taken

Table 1. *Alternative Pathway Activation by Glomerulonephritis Patient Sera*

Sample mixture‡	Complement activation fragment generation*			
	C3a	C3b/bi	C4a	C5a
	μg/ml	*%*	*μg/ml*	*ng/ml*
$NHS_1 + NHS_2 + EDTA$	1.2	0	0.35	<10
+ PBS	3.9	3	0.88	15
+ MgEGTA	4.7	6	0.94	18
NHS_1 + PSGN serum + EDTA	1.2	14	1.1	<10
+ PBS	3.8	29	1.2	<10
+ MgEGTA	3.0	31	1.3	14
NHS_1 + serum L + EDTA	2.5	10	0.80	<10
+ PBS	18.2	91	0.82	48
+ MgEGTA	27.0	95	1.2	32

* Anaphylatoxin levels were determined by a RIA and C3 conversion (C3b/bi percent) by an electrophoresis immunofixation assay as described in Materials and Methods. Values represent means of duplicate measurements.

‡ NHS and patient sera (serum from a patient with poststreptococcal glomerulonephritis or serum from patient L with MPGN) were mixed in the presence of EDTA (10 mM), buffer, or MgEGTA (5 mM MgCl2; 10 mM EGTA) and incubated for 30 min at +37°C before C activation fragment determination.

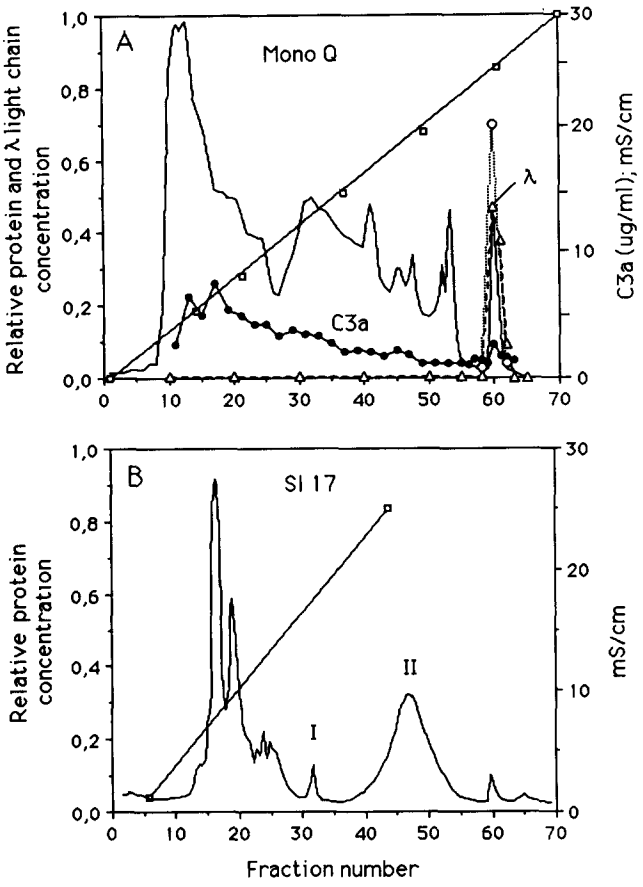

Figure 7. Purification of the complement-activating factor (λ_L) from patient serum (*A*) and urine (*B*) by anion-exchange chromatography. (*A*) Patient serum (150 μl) was applied to a Mono Q (Pharmacia) column in 10 mM diaminopropane, pH 8.3, containing 0.025% azide. Absorbance was monitored at 277 nm, and λ L chains were detected and quantitated as in Fig. 6. Purity of λ_L was analyzed by immunoelectrophoresis, SDS-PAGE, and immunoblotting assays. Functional activity was tested by determining the production of C3a in NHS either before (●) or after (○) dialysis against PBS. (*B*) Concentrated urine in 10 mM phosphate buffer was applied to a SI17 column as described in Materials and Methods. The monoclonal λ L chains bound strongly to the column and became eluted as two separate peaks (I and II).

during this period. Similar activity was also detected in samples of patient urine that was tested after pH adjustment to 7.4.

Purification and Properties of the Complement-activating Factor. When patient serum was subjected to gel filtration on a Superose 6 column in PBS, pH 7.4, the AP-activating factor was observed to elute after the IgG peak, as followed by a C3 conversion IFX assay and by C3a desArg RIA (Fig. 6). Electrophoretic analysis of the complement-activating fractions showed an atypical protein band between the α2 and β1 fractions. IFX analysis using antisera specific for Ig γ, μ, α, or δ H chains, Fd-piece, and κ and λ L chains showed that this band reacted with the anti-λ L chain antiserum but not with the other antisera. Homogeneity of the anti-λ-reactive band suggested that it is a monoclonal λ L chain. Quantitative analysis of free λ L chains in the gel filtration fractions indicated that they comigrated with the AP-activating activity (Fig. 6).

To test whether the monoclonal component induced C activation, the λ L chains were purified from patient serum as well as from plasmapheresis plasma and urine. In Mono Q anion exchange chromatography (Fig. 7 *A*), free λ L chains were found to elute as a distinct homogenous peak at a salt concentration of 0.350 M. In the presence of 0.350 M NaCl, the λ L chain–containing fractions induced only a moderate C3 activation response as measured by C3a desArg formation, and the level of spontaneous C3 conversion in the presence of the other fractions appeared to be inversely proportional to the salt concentration in the fractions. When assayed after dialysis against PBS, pH 7.4, the λ L chains induced an extensive complement activation in NHS (C3a levels up to 22 μg/ml), whereas the other fractions did not (C3a levels <3 μg/ml). The activation occurred in the presence MgEGTA (0.01 M) indicating the AP as the activation route.

SDS-PAGE of the λ L chain–containing fractions (Fig. 8 *A*, lane *1*, and *B*, lane *4*) showed a homogenous protein, which under reducing conditions (Fig. 8 *C*) migrated as a single band of ~23 kD. Ouchterlony double diffusion, immunoelectrophoresis, and immunoblotting analyses confirmed that the protein was an Ig λ L chain. For this reason, the factor was named as λ_L (L being derived from patient's family name). Protein electrophoresis showed that the isolated λ_L was a monoclonal protein migrating between the α2 and β1 fractions similarly to the monoclonal band in patient serum and urine. The concentration of free λ L chains was ~0.7 mg/ml in the patient serum. Immunoblotting analysis (Fig. 8 *B*) showed that λ_L was present both as a dimer and monomer (in an approximate ratio of 4–5:1) in patient serum and urine.

During purification of λ_L from urine (Fig. 7 *B*) two homogeneous peaks (I and II) reacting with λ L chain antisera were obtained. On SDS-PAGE under nonreducing conditions (Fig. 8 *A*) the 23-kD form was preferentially seen in peak I (lane *2*), and the 46-kD form in peak II (lane *3*), although the two forms were present in both peaks.

In cation exchange chromatography using a CM-TSK column, λ_L showed an elution pattern distinct from that of IgG (not shown). While Igs bound to this column, the monoclonal λ L chains eluted in the breakthrough fractions in 10 mM phosphate buffer, pH 6.5. λ_L bound to the CM-TSK column in 25 mM phosphate buffer, pH 5.0–5.5, and elution was achieved using a salt gradient (8.5–44 mS). Superose 6 gel filtration was used as the final step to allow separation from albumin. C3 cleaving activity was again observed to comigrate with the λ_L L chains.

Activation of the Alternative Pathway by λ_L. The purified λ_L activated the alternative complement pathway in fresh NHS (Fig. 9, *A* and *B*). Activation was dose dependent, saturable, and sensitive to ionic strength (Fig. 9, *C* and *D*). As with whole serum, activation by the purified λ_L resulted in cleavage of C3, factor B, and C5, but not of C4. Control L chains (isolated from patients with L chain myelomas), whether of λ or κ subtype, failed to activate the alternative pathway (Fig. 10).

λ_L Does Not Stabilize the Alternative Pathway C3 Convertase. Purified C components were used for studying whether

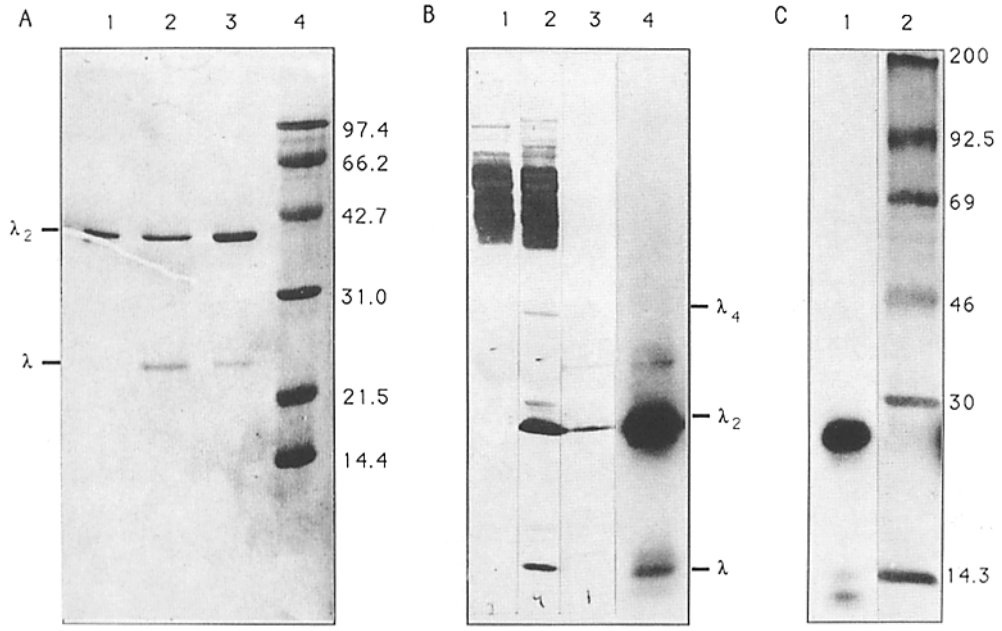

Figure 8. SDS-PAGE of λ_L purified from serum and urine and immunoblotting analysis of patient serum and urine. (*A*) Samples of serum λ_L (lane *1*, see Fig. 6 *A*), and urinary λ_L (pooled peaks I and II, lanes *2* and *3*, respectively; see Fig. 6 *B*) were run in a 12% SDS-PAGE gel under nonreducing conditions. Molecular mass markers are shown on the right. Note the higher relative amount of λ L chain monomers in peak I sample of the urinary λ_L. (*B*) Western blot analysis of patient serum and urine using anti λ-antiserum. Samples of NHS (lane *1*), patient serum (lane *2*), and urine (lane *3*) were run in SDS-PAGE (8–12% gradient gel) under nonreducing conditions. The separated proteins were transferred onto nitrocellulose and stained with peroxidase-conjugated anti-human λ antibodies. Purified radiolabeled λ_L was run as a control (lane *4*). In patient serum, λ_L appears principally as a dimer and monomer, although a trace of a tetramer (λ_4) is also present. The broad high molecular mass bands represent λ L chains of whole Igs of different isotypes. In urine, both dimers and monomers are seen. (*C*) SDS-PAGE of purified radiolabeled λ_L under reducing conditions. ^{14}C-methylated protein standard mixture is shown on the right.

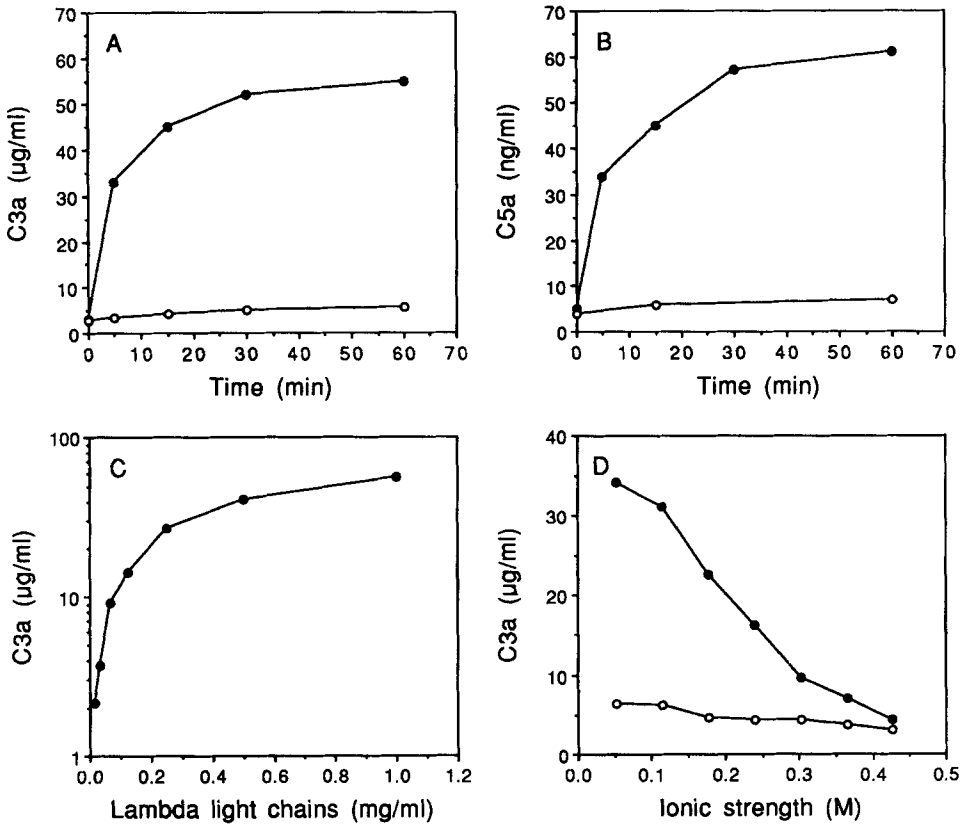

Figure 9. Time course, dose-response, and ionic strength effects on alternative pathway activation by λ_L. Activation-associated cleavage of (*A*) C3 and (*B*) C5, respectively. Purified λ_L (0.3 mg/ml, final concentration) (●) or buffer (○) were added to NHS and incubated at +37°C. Generation of C3a and C5a was quantitated by RIA. (*C*) For estimating the dose-response effect, increasing concentrations of λ_L (in 50 μl of VBS, pH 7.4) were added to 150-μl aliquots of NHS and incubated at +37°C for 30 min before quantitating C3a. (*D*) The effect of ionic strength on the alternative pathway activation response by λ_L. 100 μl λ_L (0.3 mg/ml, final) (●) or buffer (○) were mixed with NHS (150 μl) and varying concentrations of NaCl (0–1.0 M NaCl in 150 μl), and incubated at +37°C for 30 min before quantitating C3a.

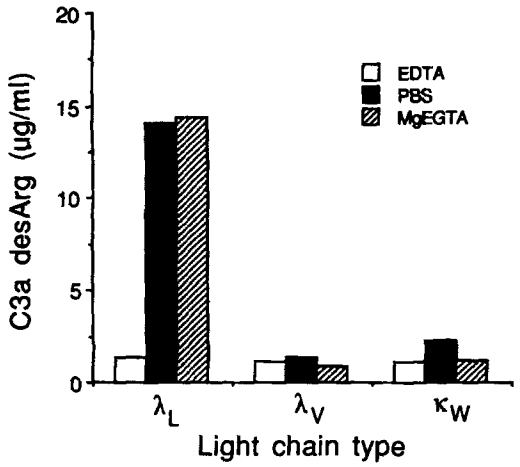

Figure 10. Complement activation by λ_L in comparison with control L chains. λ_L or control L chains (λ_V, κ_W) purified from urines of patients with L chain myelomas were incubated at 0.1 mg/ml in NHS for 30 min (+37°C) in the presence of EDTA (10 mM), PBS, or MgEGTA (5 mM $MgCl_2$, 10 mM EGTA). C3a was quantitated by RIA. C3a formation in the absence of L chains was taken as background and has been subtracted from the values shown.

λ_L binds to, and stabilizes, the AP C3 convertase, C3bBb. Properdin was used as a control stabilizing factor. The C3bBb enzyme was assembled on sheep erythrocytes (E_s). When 3.0 μg of either ^{125}I-λ_L or control λ L chains (^{125}I-λ_V) were mixed with 6 × 10^7 E_s-C3bBb cells (bearing ~30,000 C3b molecules/particle) and incubated for 30 min at +37°C, ~1.0% (λ_L) and 0.95% (λ_V) of L chains were detected bound after centrifugation through 20% sucrose. This binding did not differ significantly from binding to E_s cells alone or to E_s-C3b. No hemagglutination of λ_L-treated E_s-C3bBb cells was observed when they were mixed with polyclonal antiserum against λ L chains. Hemagglutination was positive, when the same cells were mixed with either anti-C3 (anti-C3c) or anti-factor B antiserum. After adding normal rat serum (1/15) in GVB-EDTA, no difference between the residual C3 convertase activity was observed between λ_L-treated and control L chain–treated E_s-C3bBb. As shown in Fig. 11, λ_L did not stabilize the C3bBb enzyme when present at a higher than equimolar concentration to C3b. Thus, the mechanism of alternative pathway activation by λ_L is distinct from C3 nephritic factor activity.

Binding of λ_L to Factor H. To see if the activity of λ_L could be due to its interaction with the AP control factor H, a direct binding experiment was performed. As shown in Fig. 12 *A*, radiolabeled λ_L bound to microtiter plate–fixed H, whereas no binding to C3b or BSA occurred. To see if λ_L is capable of forming fluid phase complexes with factor H, sucrose density gradient ultracentrifugation analyses were carried out. Preincubation of ^{125}I-λ_L with unlabeled factor H (or with NHS) resulted in the appearance of a new λ_L-

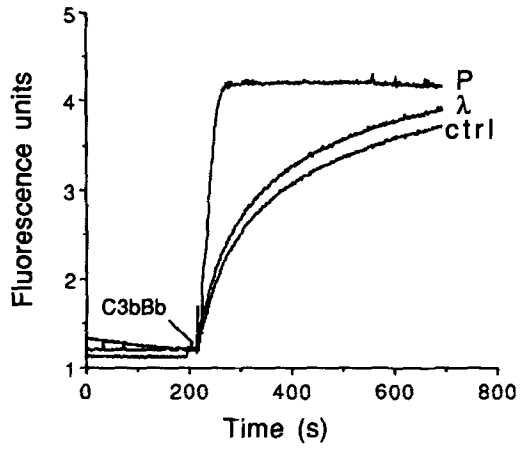

Figure 11. Lack of C3bBb-stabilizing effect of λ_L as demonstrated by a fluorescence quenching assay of C3 cleavage. Preformed C3bBb enzyme (see Materials and Methods) was added to cuvettes containing native C3 (1.0 μM), the fluorescent probe ANS (8-anilino-1-naphtalene sulphonate, 40 μM), and P (0.18 μM), λ_L (λ, 1.5 μM), or buffer (control). Follow-up of the resulting C3 cleavage to C3b shows the C3bBb stabilizing effect of properdin (highly accelerated C3 cleavage rate). λ_L does not show any significant C3bBb-stabilizing effect.

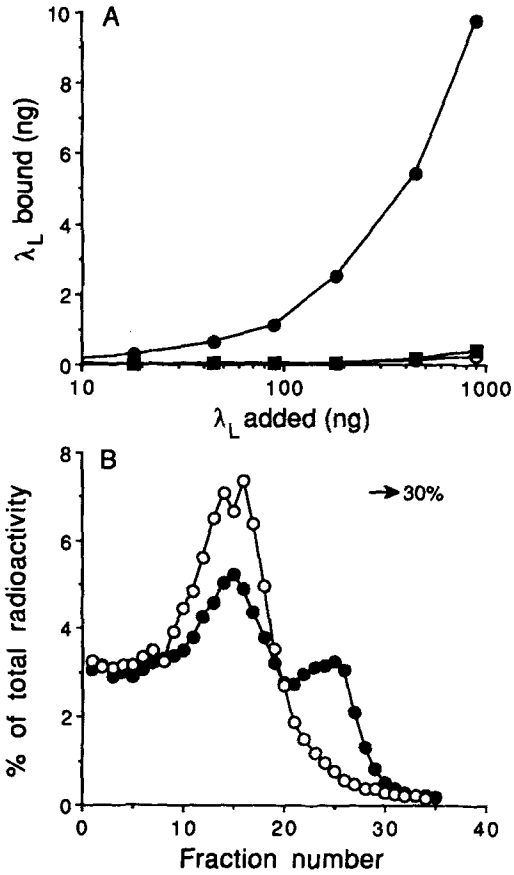

Figure 12. Binding of λ_L to factor H. (*A*) Increasing amounts of radiolabeled λ_L were allowed to bind to microtiter plate–fixed factor H (●), C3b (○) or BSA (■). Each point represents a mean of triplicate determinations. (*B*) Sucrose density gradient (10–30%) ultracentrifugation profile of ^{125}I-λ_L (final concentration, 10^{-8} M) preincubated with unlabeled factor H (●) (5.0 × 10^{-6} M) or BSA (○) (3.0 × 10^{-5} M) in PBS. Complexes of ^{125}I-λ_L with factor H correspond to a position of 7S.

containing peak apparently due to formation of ^{125}I-λ_L-H complexes (Fig. 12 *B*). Similar complexes were observed when ^{125}I-H (0.7×10^{-8} M) was added to patient serum. Regardless of the relative amounts of λ_L and H in the preincubation mixtures, the complexes migrated at a position corresponding to that of IgG (7S). This suggests that complex formation occurred primarily at an approximately 1:1 molar ratio. The 1:1 ratio was confirmed in sandwich binding experiments where neither of the two components (λ_L or H) was capable of interacting simultaneously with solid phase and fluid phase ligands (not shown).

Discussion

This report describes the detection of a novel type of alternative complement pathway-activating factor, a monoclonal λ L chain dimer, in human disease. The patient in whom the factor (λ_L) was found suffered from an atypical form of glomerulonephritis. The electron microscopic findings (dense intramembranous deposits; Fig. 1) resembled those observed in MPGN type II, the form of glomerulonephritis associated with the presence of C3 nephritic factor (C3 Nef). In addition to the characteristic intramembranous dense deposits, lesions were observed in subendothelial spaces suggesting that the glomerulonephritis in our patient was of an intermediate type between MPGN I and MPGN II.

Extensive deposition of C3, properdin, and of the terminal component components in glomeruli were seen in the kidney biopsy (Figs. 2 and 3). No deposits of Igs nor of the classical pathway components were detected. This pattern of C component deposition suggests that alternative pathway complement activation has occurred in vivo. A similar C deposition pattern has been observed in other forms of glomerulonephritis, such as in IgA nephropathy (29, 30). In the case of C3 Nef–associated glomerulonephritis, deposition of Igs in kidneys is also rare. Somewhat surprisingly, only small amounts of λ L chains were found deposited in the kidneys. The distribution pattern of λ L chains (Fig. 4) did not resemble that of C3, properdin, or C5b-9, but was similar to that usually observed in L chain disease (tubular casts and weak staining in glomerular basement membranes). The glomerular basement membrane deposition of λ L chains was somewhat similar to that of the alternative pathway control factor H, which appeared in a linear fashion along the basement membranes rather than as coarse deposits (Fig. 3 *C*). These observations, together with the demonstration of an efficient complement-activating capacity in the patient serum (Fig. 5 and Table 1), indicate that AP activation has been initiated in the fluid phase and not at the sites of "activator" deposition. It is likely that C3b, after its formation in the fluid phase, has become randomly attached to glomerular and vascular endothelial cell membranes with consequent deposition of further C components. The similar patterns of C component deposition and ultrastructural changes in our patient and in MPGN type II with C3 Nef suggest that the pathological changes in glomeruli predominantly result from AP activation instead of being directly caused by the abnormal Igs.

Initially, the complement-activating capacity in the patient serum was suspected to be due to C3 nephritic factor until it was observed that in gel filtration (Fig. 6) and in anion exchange chromatography (Fig. 7 *A*) the activity comigrated with free λ L chains. Subsequent purification of the complement-activating factor (Fig. 7) and demonstration of its reactivity with anti–human λ L chain antisera further indicated its relationship to λ L chains. The AP-activating factor did not react with anti-Fd antiserum, nor with anti–κ or anti–H chain antisera. The homogeneous appearance of the λ L chains in agarose electrophoresis and in immunofixation demonstrated their monoclonality. Analysis of the activating factor by SDS-PAGE suggested that it is a dimer composed of two monoclonal, and probably identical, disulphide-linked 23-kD polypeptides. These results establish the AP-activating factor as a monoclonal λ L chain dimer (λ_L).

Immunoblotting and immunofixation experiments with anti-λ L chain antisera (Fig. 8) demonstrated that λ dimers and monomers were the principal monoclonal components in patient serum and urine. There was no evidence for the presence of a circulating "parent" full size Ig carrying monoclonal λ L chains in patient blood. During a follow-up of 2 yr, the patient showed no evidence of developing myeloma. In contrast, the levels of monoclonal λ L chains gradually declined in her blood and reappeared only slowly after repeated plasmapheresis. Thus, λ_L is probably the product of a benign clone of plasma cells producing λ L chains.

The isolated λ_L showed similar C activation kinetics as the whole patient serum, and activation of C by either patient serum or purified λ_L resulted in cleavage of C3, factor B, and C5, but not of C4. The small molecular mass discriminates λ_L from C3 Nef, which in most cases has been shown to be IgG (9, 10). The fact that λ_L differs from IgG in molecular composition does not exclude the possibility that λ_L would act similarly as C3 Nef in activating the AP, i.e., by stabilizing the AP C3 convertase, C3bBb. This possibility was tested by both conventional C3bBb binding and stabilization experiments and by a novel fluorimetric test (Fig. 11; reference 28). The validity of the fluorometric test for C3bBb stabilization was confirmed by using P as a positive control. The results indicated that λ_L did not bind to or stabilize the alternative pathway C3 convertase enzyme, C3bBb. Thus, in addition to molecular properties, λ_L and C3 Nef differ from each other also by functional criteria.

Some of the early descriptions of C3 Nef were controversial as to its immunochemical nature (e.g., reference 31). Bartlow et al. (32) have described a case of non-Ig C3-activating factor in MPGN, although the exact nature of the factor remained unsolved. In light of the present study, this factor could functionally be distinct from C3 Nef and perhaps resemble λ_L in its mechanism of action. Without sensitive techniques for detection of monoclonal proteins, λ_L-like factors are easily missed.

The lack of C3bBb-stabilizing effect of λ_L indicates that other mechanism(s) for AP activation by circulating serum factors, especially in association with glomerulonephritis, exist. A clue to the mechanism of action of λ_L was provided by

the observation that it binds to factor H (Fig. 12). This binding appeared to occur at a molar 1:1 ratio as both the ^{125}I-λ_L-H (Fig. 12 *B*) and ^{125}I-H-λ_L complexes migrated at a position of 7S. As it has been observed that L chain dimers derived from IgG1-class Igs (anti-Mcg) may retain approximately the same binding properties as the whole parent molecules (33), it is possible that λ_L has an antigen-binding site via which it reacts with factor H. Activation of the AP results if λ_L, via this direct antibody activity or by other physicochemical means, blocks the activity of the AP control factor H, which under normal conditions functions as a negative regulator of the AP activation system. A functionally similar situation would be expected to occur in cases of inherited deficiency of factor H. A report by Levy et al. (34) described factor H deficiency in two brothers. Both of the patients suffered from glomerulonephritis, which ultrastructurally and immunohistochemically showed a striking resemblance to our patient. Thus, the absence or functional neutralization of the alternative pathway control factor H, by allowing increased alternative pathway turnover in the fluid phase, appears to predispose to the development of glomerulonephritis.

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