# Neoantigen of the Polymerized Ninth Component of Complement

# CHARACTERIZATION OF A MONOCLONAL ANTIBODY AND IMMUNOHISTOCHEMICAL LOCALIZATION IN RENAL DISEASE

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ABSTRACT A monoclonal antibody to a neoantigen of the C9 portion of the membrane attack complex (MAC) of human complement has been developed and characterized. The distribution of this neoantigen was assessed by indirect immunofluorescence microscopy in nephritic and nonnephritic renal diseases. The antibody (Poly C9-MA) reacted on enzyme-linked immunosorbent assay (ELISA) with a determinant in complement-activated serum that was undetectable in normal human serum (NHS). Zymosan particles incubated in NHS had positive immunofluorescent staining with Poly C9-MA; however, binding of Poly C9-MA was not observed with zymosan particles incubated in sera deficient in individual complement components C3, C5, C6, C7, C8, or C9. Reconstitution of C9-deficient sera with purified C9 restored the fluorescence with Poly C9-MA. Poly C9-MA reacted positively by ELISA in a dose-dependent manner with purified MC5b-9 solubilized from membranes of antibody-coated sheep erythrocytes treated with NHS but not with intermediate complement complexes. Poly C9-MA also reacted in a dose-dependent manner on ELISA and in a radioimmunoassay with polymerized C9 (37°C, 64 h) (poly C9) but not with monomeric C9. Increasing amounts of either unlabeled poly C9 or purified MC5b-9 inhibited the <sup>125</sup>I-poly C9 RIA in an identical manner. These studies demonstrate that Poly C9-MA recognizes a neoantigen of C9 common to both the MAC and to poly C9. By immunofluorescence, Poly C9-MA reacted minimally with normal

kidney tissue in juxtaglomerular loci, the mesangial stalk, and vessel walls. Poly C9-MA stained kidney tissue from patients with glomerulonephritis in a pattern similar to that seen with polyclonal anti-human C3. In tissue from patients with nonnephritic renal disease-diabetes, hypertension, and obstructive uropathy-Poly C9-MA was strongly reactive in the mesangial stalk and juxtaglomerular regions, tubular basement membranes, and vascular walls. Poly C9-MA binding was especially prominent in areas of advanced tissue injury. Poly C9-MA frequently stained loci where C3 was either minimally present or absent. These studies provide strong evidence for complement activation not only in nephritic but also in nonnephritic renal diseases.

# INTRODUCTION

The complement system has been implicated in the pathogenesis of experimental and human renal disease by virtue of changes in serum levels and by the demonstration of complement components in diseased tissue (1). One of the mechanisms of complement-induced membrane damage is mediated through the assembly of terminal complement components into the membrane attack complex (MAC)<sup>1</sup> MC5b-9 (2). The

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<sup>&</sup>lt;sup>1</sup> Abbreviations used in this paper: DOC, sodium-desoxycholate; EA, antibody-coated sheep erythrocytes; EAC, complement-coated EA; ELISA, enzyme-linked immunosorbent assay; GBM, glomerular basement membrane; LPS, endotoxin lipopolysaccharide; MAC, membrane attack complex; MPGN, membranoproliferative glomerulonephritis; NHS, normal human serum; poly C9, polymerized C9; Poly C9-MA, monoclonal antibody to a neoantigen of C9; SLE, systematic lupus erythematosus; VBS, veronal-buffered saline.

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five MAC precursor proteins assemble after cleavage of complement component C5 by classic or alternative complement pathway activation (3). Once formed, the MAC binds with high affinity to phospholipid binding sites on cell membranes (4). Presumably, the MAC is capable of physically inserting itself into and then reorganizing membrane lipid bilayers (5, 6). In the process of assembly of the MAC, the terminal complement components undergo conformational changes exposing neoantigenic structures in C5b6 and in C5b67, recognized by previously described polyclonal antibodies (7). The C5b67 complex is membrane bound, allowing for subsequent C8 and C9 attachment (6). Recently, Podack et al. (8) have demonstrated that polymerization of C9 induced by cell-bound C5b-8 or by prolonged incubation of monomeric C9 at 37°C forms tubular structures with an ultrastructural appearance that resembles that of the isolated MAC. Additionally they have produced a polyclonal antiserum to a neoantigen(s) on polymerized C9 (poly C9) that is similar to the antigen(s) present on isolated MAC (9).

Although the MAC has been recognized for several years on lymphocytes (10), polymorphonuclear leukocytes (11), and platelets (12), its presence in diseased tissues has been appreciated only recently. Biesecker et al. (13, 14) demonstrated that a polyclonal antibody to MAC fixes to immune deposits in the kidney and skin of patients with systemic lupus erythematosus (SLE).

In the course of studies of monoclonal antibodies to human renal basement membranes, we found and characterized a monoclonal antibody (Poly C9-MA) to a neoantigen present in the C9 portion of the MAC. In normal kidney tissue, this antigen is present in small vessels and minimally within the glomerular stalk, whereas in kidney tissue from a variety of diseases there is extensive deposition of this neoantigen of the MAC.

#### METHODS

Buffers. The following buffers were used: Veronal-buffered saline (VBS); VBS containing 0.15 mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub> (VBS<sup>++</sup>); phosphate-buffered saline (PBS) (0.15 M NaCl, 0.01 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4); 0.5 PBS (0.5 M NaCl, 0.01 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4); PBS-EDTA (PBS with 10 mM EDTA); BEP buffer (5 mM Na borate with 10 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, pH 8.8) (15); enzymelinked immunosorbent assay (ELISA) coating buffer (0.1 M Na<sub>2</sub>CO<sub>3</sub>, pH 9.6); ELISA wash buffer (0.5 PBS with 0.05% Tween 20, Sigma Chemical Co., St. Louis, MO); citrate phosphate buffer (0.5 M citric acid and 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 5.0); and Tris-hydroxymethyl aminomethane-buffered saline (Trisbuffered saline; 0.10 M Tris, 0.15 M NaCl, pH 7.4).

Complement components and reagents. The following human complement proteins were purified as described previously: C5 (16), C6 (17), C5b6 (18), C7 (17), C8 (19), and C9 (20). C5b67 complexes were formed by reacting 100  $\mu$ g of C5b6 with 50  $\mu$ g of C7 for 30 min at 37°C. Poly C9 was

prepared by incubating purified C9 at 1 mg/ml for 64 h at 37°C in Tris-buffered saline containing 25  $\mu$ g soybean trypsin inhibitor (8). Electron microscopic analysis of the poly C9 using a negative staining technique showed tubular structures identical to those previously reported (8). Analysis of the purified poly C9 by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was conducted on a 2.5-10% polyacrylamide gradient as previously described (9). The purity of the preparation is demonstrated in Fig. 1. By densitometric scanning in different preparations, 22-50% of the material was present in the poly C9 form. Some experiments were performed with functionally pure C8 and C9 (Cordis Laboratories, Inc., Miami, FL).

Zymosan (Becton-Dickinson and Co., Orangeburg, NY), cobra venom factor (Naja Naja) (Cordis Laboratories), and endotoxin lipopolysaccharide (LPS) from *Escherichia coli* 026:B6 (lot 613126, Difco Laboratories, Detroit, MI) were used as complement activators. Human sera deficient in specific complement components, C1r, C2, C3, C5, C6, C7, C8 ( $\alpha, \gamma$  chain), C8 ( $\beta$  chain), and C9 were characterized by component analysis as previously described (21). An additional C9 deficient serum was a kind gift from Dr. Shinya Inai, Osaka, Japan. Single donor normal human cryoprecipitate (22) was obtained from the University of Minnesota Blood Bank.

Collagen types I, III, IV, and V were isolated from human placenta (23), but without reduction, carboxymethylation, or repeated pepsin digestion. Laminin was a gift of Dr. Hynda Kleinman (National Institute of Dental Research), and fibronectin was a gift of Dr. Leo Furcht (University of Minnesota).

Development of the monoclonal antibody Poly C9-MA. Collagenase-digested normal human glomerular basement membrane (GBM) was prepared from kidney tissue that was obtained at the time of autopsy after accidental death (24, 25). Balb/c female mice were immunized by an intraperitoneal injection of 100  $\mu$ g of GBM homogenized in complete Freund's adjuvant (Gibco Laboratories, Grand Island, NY), followed by two immunizations in PBS at intervals of 14 d.

Hybridization and cloning. 4 d after the final immunization, spleen cells in Dulbecco's modified Eagle medium



FIGURE 1 SDS-polyacrylamide gel electrophoresis (2.5-10%) gradient) of purified C9 (lane I); poly C9 (lane II), purified C5 (lane III), and molecular markers (lane IV). By densitometric scanning of lane II, ~25% of purified C9 polymerized into poly C9.

(Microbiological Associates, Walkersville, MD) were fused with the Balb/c myeloma cell line P3-Nsl-l-Ag4-l (obtained from Dr. Tucker LeBien, University of Minnesota) in polyethelene glycol (PEG 1000, J. T. Baker Chemical Co., Phillipsburg, NJ) (26). The cells were grown in 96-well microtiter plates (Costar, Cambridge, MA) in HAT medium containing Littlefield's concentrations of hypoxanthine, aminopterin, and thymidine (27). Supernatants from visible hybrid colonies were screened for antibody by indirect immunofluorescence on normal human kidney. Colonies of interest were subcloned by limiting dilution, then grown to culture volume, and injected into pristane-primed Balb/c female mice. Ascites fluid was harvested 7 to 14 d after injection (28). The supernatant of cell cultures was stored at  $-70^{\circ}$ C.

Immunofluorescent studies. Tissue was snap frozen in isopentane precooled in liquid nitrogen and sectioned at 4  $\mu$ M in a Lipshaw cryostat (Lipshaw Manufacturing, Co., Detroit, MI). After air-drying, sections were fixed in acetone for 10 min, washed three times with PBS, overlaid with  $\sim 20 \ \mu$ l of an appropriate dilution of monoclonal ascites fluid, or goat antibody to human C3, C5, C6, C7, C8, or C9 (Miles Laboratories, Elkhart, IN). Each goat antiserum was absorbed with its respective complement-deficient serum before use. Monoclonal antibodies were reacted with human plasma-absorbed fluorescein-isothiocyanate-conjugated goat anti-mouse Ig ( $\gamma$ -,  $\mu$ -,  $\alpha$ -chain specific; Cappel Laboratories Inc., Cochranville, PA) and the complement component antibodies were reacted with human plasma-absorbed fluorescein-isothiocyanate-conjugated rabbit anti-goat Ig ( $\gamma$ -,  $\mu$ -,  $\alpha$ -chain specific, Cappel Laboratories) (29). The distribution and intensity of immunofluorescence was scored on a semiquantitative scale of trace, moderate, and strong fluorescence. Class-specific reagents to mouse immunoglobulin heavy and light chains (Bionetics Laboratory Products, Kensington, MD) were used after the application of the monoclonal antibody to determine Ig class specificity. Immunofluorescent studies with Poly C9-MA were performed on lymphocytes, monocytes, and polymorphonuclear leukocytes, which were obtained by Ficoll-Hypaque gradient cen-

trifugation as previously reported (30). Zymosan immunofluorescence assay. Zymosan was added to VBS at 1 mg/ml and boiled at 100°C for 30 min. The sample was centrifuged at 4°C and at 300 g for 5 min, resuspended and washed three times with cold VBS++ (31). The zymosan particle suspension (100  $\mu$ g in 100  $\mu$ l) was incubated at 37°C in 100 µl normal human serum (NHS), heatinactivated NHS (prepared by preincubation at 56°C for 30 min), NHS containing 10 mM EDTA, or a specific complement component deficient serum, C1r, C2, C3, C5, C6, C7, C8 ( $\alpha$ - $\gamma$  chain), C8 ( $\beta$  chain), or C9. The zymosan particles were washed three times with cold VBS as above, air dried on glass slides, and stained by indirect immunofluorescence with goat antisera to human C3, C5, C6, C7, C8, or C9 (each absorbed with boiled zymosan particles) and Poly C9-MA. In some studies, a monoclonal antibody that reacts with human basement membrane (MBM10) of the same subclass as Poly C9-MA  $[IgG_1, \kappa]$  was also used. C8- or C9-deficient human serum (100  $\mu$ l) was reconstituted with the respective functional component, C8 or C9 (400 hemolytic units), and incubated with zymosan particles for 30 min at 37°C, washed, and prepared for immunofluorescence microscopy.

ELISA studies (32, 33). Antigens were incubated in 96well microtiter plates (Costar) with ELISA coating buffer or with PBS in a moist chamber at room temperature for 3 h, then overnight at 4°C. The plates were washed three times with ELISA wash buffer and incubated at room temperature for 1 h with 100  $\mu$ l of monoclonal antibody or goat antisera specific for human complement components (C5, C6, C7, C8, or C9) diluted in PBS. After three washes, the plates were incubated for 30 min at room temperature with human plasma-absorbed peroxidase-labeled goat anti-mouse Ig ( $\gamma$ ,  $\mu$  chain specific) (Tago, Inc., Burlinghame, CA) or human plasma-absorbed rabbit anti-goat IgG (heavy and light chain specific) (Cappel Laboratories). The substrate for the peroxidase reaction was a solution of 0.04% ortho phenylenediamine dihydrochloride (Eastman Kodak Co., Rochester, NY) in citrate phosphate buffer with 0.012% hydrogen peroxide (Mallinckrodt, Inc., Paris, KY). Optical density was read at 30 min at 450 nm with a Titertek Multiscan (Flow Laboratories, Helsinki, Finland). Positive results were determined (a) by comparison of the absorbency of the test antiserum with that of an unrelated control antiserum and by comparison of the test antiserum on the test antigen and an unrelated control antigen and (b) by statistical comparison (paired t test).

MC5b-9 complex isolation. MC5b-9 complexes were isolated from sheep erythrocytes as described by Ware et al. (15). Briefly, antibody-sensitized sheep erythrocytes (EA) were incubated with human serum diluted in VBS++ for 60 min at 37°C. The complement-coated EA membranes (EAC) were washed three times in BEP buffer, centrifuged at 27,000 g and 4°C for 20 min, solubilized with Triton X-100 (Sigma Chemical Co.) or Zwittergent ionic detergent 3-12 (SB12, Calbiochem-Behring, La Jolla, CA) for 30 min at room temperature. The detergent-to-protein weight ratio was 40:1. The mixture was centrifuged at 27,000 g for 20 min and the supernatant was concentrated on a YM-30 membrane (Amicon Co., Lexington, MA), and stored at 4°C. Purified MC5b-9 was obtained from EAC membranes solubilized in SB12 and isolated by Bio-Rad A 15 M chromatography (Bio-Rad Laboratories, Richmond, CA) as previously reported (15). Additional purified MC5b-9 was kindly provided by Dr. William Kolb (San Antonio, TX).

Reconstitution experiments were performed with EAC1-7 cells prepared as follows: 1.0 ml of C8-deficient serum was incubated with  $1.6 \times 10^9$  EA for 60 min at 37°C and then washed six times with cold VBS++ to remove residual serum components. The absence of C9 on the EAC1-7 intermediates was demonstrated by the lack of aggregation of these cells by anti-human C9 antiserum. EACI-7 intermediates were then incubated at 37°C for 60 min with 2,000 hemolytic units of C8 alone or 2,000 hemolytic units of both C8 and C9, or 2,000 hemolytic units of C9 alone. The EAC membranes were washed, solubilized, and concentrated by ultrafiltration as described above. Unlysed EA cells (those incubated with heat-inactivated serum or C8-deficient serum) were lysed during the BEP buffer washes. A similar experiment was performed in which solubilized EAC membranes were obtained from EA incubated in C9-deficient serum alone, or C9-deficient serum reconstituted with 2,000 hemolytic units of C9.

Serum C5b-9 (SC5b-9) was made by incubating NHS with LPS (300  $\mu$ g/ml of serum) or cobra venom factor (20 U/ml of serum) for 1 h at 37°C. These sera were placed directly on microtiter plates for ELISA. Binding to the plates was similar whether antigens were incubated in coating buffer or in PBS.

Analysis of reactivity of Poly C9-MA with poly C9 and with monomeric C9. The interaction of Poly C9-MA with poly C9 and with monomeric C9 was investigated by ELISA and by a radioimmunoassay (RIA). For the RIA, C9 was labeled with <sup>125</sup>I by a lactoperoxidase procedure (34) at 4°C for 4 h. The specific activity of C9 was 110,000 cpm/µg protein. Radiolabeled poly C9 was prepared from monomeric <sup>125</sup>I-C9, with a sp act of 10,400 cpm/ $\mu$ g protein. The specific activity of monomeric C9 was made 10-fold greater than that of poly C9 to maximize the sensitivity of the reaction of Poly C9-MA with monomeric C9. To obtain monomeric C9 free of possible aggregates that might have formed during its purification and storage, 0.22 mg of <sup>125</sup>I-C9 was mixed with 2.4 mg of bovine serum albumin (BSA, Sigma Chemical Co.), applied to a  $1.5 \times 100$ -cm Sephadex G-100 column (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with PBS. Fractions containing the monomeric component were used directly in the immunoassays. The presence of functionally active C9 in these fractions was eAC1-7 in the presence of limiting amounts of C8.

200  $\mu$ l of immunobeads bearing rabbit anti-mouse Ig (Bio-Rad Laboratories), were incubated with 5  $\mu$ l of Poly C9-MA or the control monoclonal MBM10 for 1 h at room temperature. The beads were washed three times with PBS containing 0.05% Tween and once with PBS at 4°C. The beads were then transferred to polypropylene tubes (Walter Sarstedt, Inc., Princeton, NJ) precoated with 1% BSA to prevent nonspecific adherence of <sup>125</sup>I-labeled material to the walls of the tube. To saturate the beads fully, 1  $\mu$ g of radiolabeled monomeric C9 or poly C9 was incubated at room temperature for 1 h with the immunobeads, which were then washed as above, and the radioactivity was measured in a gamma counter.

To prepare a uniform <sup>125</sup>I-poly C9 standard, <sup>125</sup>I-poly C9 was incubated and stored in PBS-1% sodium-desoxycholate (DOC, Fischer Scientific Co., Fairlawn, NJ), thereby decreasing the aggregation of poly C9. Incubation in DOC did not change the dose-response binding characteristics of <sup>125</sup>I-poly C9 to Poly C9-MA. The DOC-treated material was applied to a Bio-Rad A15 M (0.9  $\times$  42 cm) column and two major peaks eluted with PBS at a flow rate of 5 ml/h. Fractions from the first peak that demonstrated positive binding to Poly C9-MA by RIA were pooled and stored at 4°C in PBS-1% DOC.

Dose-response curves of <sup>125</sup>I-poly C9 or <sup>125</sup>I-C9 were determined by adding increasing amounts of either material (protein concentrations of 1  $\mu$ g/ml) to 50  $\mu$ l of immunobeads that were incubated with 10  $\mu$ l of a dilution of Poly C9-MA supernatant. The total amount of labeled material added was measured, the beads washed, and the amount of bound radioactivity counted.

Inhibition of the <sup>125</sup>I-poly C9 RIA by poly C9 and purified MC5b-9. Inhibition of <sup>125</sup>I-poly C9 binding to the immunobeads was accomplished using unlabeled cold poly C9 or purified MC5b-9. Immunobeads were incubated with Poly C9-MA supernatant, washed as above, and transferred in PBS-1% DOC to BSA-coated polypropylene tubes. Unlabeled poly C9 (protein concentration of 100  $\mu$ g/ml) that had been stored in PBS-1% DOC was then added to the immunobeads in 10- $\mu$ l increments. After 1 h of incubation on a rotating platform at room temperature, a constant amount of <sup>125</sup>I-poly C9 was added and total radioactivity measured. After 10 min of incubation at room temperature, the beads were washed and the bound counts determined. In other experiments, 10- $\mu$ l increments of purified MC5b-9 (protein concentration of 100  $\mu$ g/ml) were added under exactly the same conditions instead of unlabeled poly C9.

Patients. Kidney tissue was selected from two groups of patients. One group included 16 patients with glomerulo-nephritis: membranoproliferative glomerulonephritis (MPGN) type I (n = 3), MPGN type II (n = 2), membranous nephropathy (n = 3), SLE, n = 3), IgA nephropathy (n = 3), and anaphylactoid purpura nephritis (n = 2). Tissue

was obtained by percutaneous renal biopsy from 14 of these patients with serum creatinine levels ranging from 0.7 to 2.3 mg/dl. In two other patients, both with MPGN type II, the kidney tissue was obtained at the time of nephrectomy, before renal transplantation. The second group of patients included 17 patients with diseases other than glomerulonephritis: diabetes mellitus (n = 7), hypertension (n = 3), obstructive uropathy (n = 3), congenital dysplasia (n = 2), and amyloidosis (n = 2). Kidney tissue was obtained by percutaneous renal biopsy in eight patients (four with diabetes mellitus, and one each with hypertension, obstructive uropathy, congenital dysplasia, and amyloidosis); the serum creatinine of these patients ranged between 0.6 and 2.1 mg/dl. Kidney tissue from the other nine patients in the nonnephritic group was obtained at the time of nephrectomy, before renal transplantation. All tissues were evaluated and characterized by light and electron microscopy as previously described (35-37). Routine immunofluorescent studies were carried out with polyclonal antisera to human IgG, IgM, IgA, Clq, C3, C4, properdin, albumin, and fibrinogen (36. 38). The immunohistochemical localization of immunoglobulins and other antigens will not be reported in this paper. There was a good correlation between the deposition of immunoglobulin and C3 in kidney tissue from patients with glomerulonephritis, except for those with MPGN. In the latter disease, the deposition of C3 was more extensive than that of immunoglobulin (37). In tissue from patients with nonnephritic disease, a similar discrepancy was obtained between the deposition of C3 and that of immunoglobulin, as previously reported (36, 38).

### RESULTS

Initial studies with Poly C9-MA. The monoclonal antibody (Poly C9-MA)  $[IgG_1, \kappa]$  was developed after immunization with collagenase-digested normal human basement membranes. However, Poly C9-MA did not react on ELISA with either the collagenase-digested GBM material used for immunization or the known constituents of renal extracellular matrices: collagen type I, III, IV, V, fibronectin, and laminin. Each antigen was tested with an appropriate positive control antiserum. Immunohistochemical studies on human kidney specimens from patients with glomerulonephritis suggested a fluorescence pattern of Poly C9-MA similar to that observed for anti-C3 antisera. However, extensive absorption of Poly C9-MA with normal human plasma failed to diminish immunofluorescence. By ELISA, the optical density of the reaction of Poly C9-MA with 10 different samples of untreated normal human serum was 0.053±0.02 (mean±SD) (Fig. 2). When each of these serum samples was activated with LPS, an OD of  $0.193 \pm 0.11$  was seen (P < 0.005). This difference was significantly inhibited by heat inactivation of the serum before addition of LPS (0.076 $\pm$ 0.05, P < 0.01). A similar positive interaction was seen when serum was activated with cobra venom factor; no binding was observed with cryoprecipitate as well as with serum treated with 0.02 M EDTA before LPS activation.



FIGURE 2 Comparison by ELISA of the interaction of Poly C9-MA with 10 different samples of normal human serum (OD [mean±SD], 0.053±0.02) and with the same samples of human sera activated with LPS (OD [mean±SD] 0.193±.11) (P < 0.005).

Immunofluorescent reactivity of Poly C9-MA with complement-treated zymosan particles. Because Poly C9-MA appeared to bind to an antigen expressed only after complement activation, a more specific reaction with complement components was investigated on complement-treated zymosan particles. As shown in Table I, Poly C9-MA recognized an antigen present on zymosan incubated in NHS. This antigen was not detected after heat inactivation or divalent cation chelation of serum. However, Poly C9-MA reacted with zymosan particles incubated with sera deficient in C1r and C2, but not with sera deficient in any of the individual components C3 to C9. Reconstitution of C8or C9-deficient sera with the functionally purified missing component restored Poly C9-MA fluorescence (Fig. 3, Table I). These data indicate that Poly C9-MA recognized an antigen formed by the reaction of C9 with C5b-8.

Interaction of Poly C9-MA with MC5b-9, poly C9 and other complement components analyzed with ELISA. Poly C9-MA reacted positively by ELISA with either 1  $\mu$ g of poly C9 or purified MC5b-9; this latter complex was obtained from EAC membranes by solubilization with SB12 and gel filtration chromatography (Table II). There was positive reactivity of polyclonal anti-human C9 with both poly C9 and MC5b-9 complexes, whereas the unrelated IgG1 monoclonal antibody MBM10 had no reactivity (Table II). When Poly C9-MA was reacted with 10-fold serial dilutions of MC5b-9 or poly C9 (initial protein concentrations of 100  $\mu$ g/ml), there was a similar linear decrease in reactivity (Fig. 4). The slopes of the MC5b-9 and poly C9 dose-dependent relationships as determined by linear regression analysis were -0.12 and -0.11, respectively. There was no binding of Poly C9-MA to purified C5, C8, C9, and to the intermediates C5b6 and C5b67 (Table II). Because of the nature of our ELISA, inhibition studies could only be carried out with low

Sera used for zymosan incubation	Indirect immunofluorescent reactivity of serum-treated zymosan particles with complement antisera								
	Anti-C3	Anti-C5	Anti-C6	Anti-C7	Anti-C8	Anti-C9	Poly C9-MA*		
NHS	+	+	+	+	+	+	+		
Heat-inactivated or NHS + 10 mM EDTA	_	-	-	-	-	_	-		
Complement-deficient sera									
Clr	+	+	+	+	+	+	+		
C2	+	+	+	+	+	+	· +		
C3	-	-	-	-	-	-	_		
C5	+	-	-	-	-	_	-		
C6	+	+	-	-	-	-	-		
C7	+	+	+	_	-	-	-		
C8 $\alpha - \gamma$ chain	+	+	+	+	-	_	_		
C8 $\beta$ chain	+	+	+	+	-		-		
C9	+	+	+	+	+	_	-		
C8-deficient sera + C8							+		
C9-deficient sera + C9							+		

 TABLE I

 Characterization of the Reactivity of Poly C9-MA with Zymosan Treated with NHS or Complement-deficient Sera

 $^{\circ}$  As a control, no reactivity was demonstrated using an unrelated IgG<sub>1</sub> monoclonal antibody (MBM10) with specificity for human basement membrane.



FIGURE 3 Immunofluorescence of opsonized zymosan particles stained with Poly C9-MA (A). When zymosan particles were incubated in C9-deficient serum reconstituted with functionally purified C9, positive immunofluorescence was observed.  $\times$  480. (B) Zymosan particles incubated in C9-deficient serum alone failed to react with Poly C9-MA.  $\times$  480.

concentrations of Poly C9-MA. Therefore a 1:400 dilution of Poly C9-MA supernatant yielding an OD of 0.10 was selected. 1  $\mu$ g of purified MC5b-9 was incubated with Poly C9-MA for 1 h at room temperature and overnight at 4°C. The mixture was then centrifuged at 27,750 g for 30 min. The OD of the absorbed Poly C9-MA was 0.03, which was identical to that of the control using the peroxidase-labeled antibody without the primary antibody.

To verify the requirement of C9 for recognition of Poly C9-MA, EAC1-7 and EAC1-8 intermediates were formed by incubating EA in complement-defi-

	Reactivity of antibodies with various substrates by ELISA								
	Poly C9-MA	Anti-C5	Anti-C8	Anti-C9	MBM10*				
			OD units						
Purified antigens									
Poly C9‡	0.42			1.42	0.09				
C5b-9§	0.62			1.29	0.09				
C5b6	0.05	1.59			0.09				
C5b67	0.06	1.59			0.09				
C5	0.09	1.69			0.14				
C8	0.14		1.56						
C9	0.09			1.70	0.12				
Solubilized membranes									
MC5b-9 <sup>II</sup>	0.44			1.42	0.05				
MC5b-8	0.11				0.08				
MC5b-7	0.08				0.06				
MC5b-8 + C9	0.23				0.08				
NHS	0.07				0.07				
Type I collagen	0.07	0.16	0.11	0.26	0.08				

TABLE II Binding of Poly C9-MA to Poly C9 and MC5b-9 but Not to Other Complement Component Intermediates

\* Monoclonal antibody (IgG<sub>1</sub>) reactive with basement membranes.

 $\ddagger 1 \mu g$  of poly C9 was used for each test.

§ 1  $\mu$ g of purified MC5b-9 was used for each test.

<sup>11</sup> Solubilized membranes obtained from EA incubated in heat inactivated serum reacted with Poly C9-MA yielded an OD of 0.02 (MBM10, 0.04).



FIGURE 4 Linear regression analysis of Poly C9-MA binding on ELISA to serial 10-fold dilutions of Poly C9 and MC5b-9 demonstrated similar linear relationships. The initial protein concentrations (C) of poly C9 and MC5b-9 were 100  $\mu$ g/ml. The slope of the Poly C9 relationship was -0.11, while that of MC5b-9 was -0.12 (r = 0.95 for both reactions).

cient sera. Because of limited amounts of complementdeficient sera, purification of these complexes by gel filtration chromatography was not feasible. Membranes solubilized by Triton X-100 from EA cells incubated in NHS reacted with Poly C9-MA in a manner similar to that of purified MC5b-9 (Table II). As a control, solubilized membranes that had been incubated in heat-inactivated serum did not bind to Poly C9-MA. EAC membranes bearing C1-7 were produced by incubating EA in C8 deficient serum. EAC1-8 was formed by the addition of functionally purified C8 to the extensively washed EAC1-7, resulting in hemolysis. A control was performed by adding C9 alone to the EAC1-7 cells without the addition of C8. EAC membranes were then solubilized with Triton X-100 and reacted on ELISA with Poly C9-MA and the control monoclonal antibody MBM10. As shown in Table II, Poly C9-MA reacted with EAC membranes containing MC5b-9 obtained after incubation with fully reconstituted sera and not with EAC1-7 or EAC1-8 solubilized membranes. Similar results were obtained when EA were incubated in C9-deficient serum or C9-deficient serum reconstituted with functionally pure C9.

Interaction of Poly C9-MA with poly C9, monomeric C9, and MC5b-9 determined by RIA. In a solid-phase RIA system, Poly C9-MA was incubated with <sup>125</sup>I-poly C9 or <sup>125</sup>I-monomeric C9. In the first experiment, excess poly C9 was used to fully saturate the binding capacity of the Poly C9-MA attached to the immunobead. Poly C9-MA demonstrated a specific uptake of 0.174  $\mu$ g of <sup>125</sup>I-poly C9. MBM10 served as a negative control and gave an uptake of 0.008  $\mu$ g of <sup>125</sup>I-poly C9. Under the same conditions, the uptake of monomeric C9 by Poly C9-MA was  $0.002 \ \mu g$  of  $^{125}I$ -C9, which is only 1.4% of the uptake of poly C9. When  $^{125}I$ -poly C9 was added in increasing amounts, a dose-response curve was obtained (Fig. 5 A). Increasing the amounts of  $^{125}I$ -C9 did not induce binding to Poly C9-MA.

When increasing amounts of either unlabeled poly C9 or purified MC5b-9 were added to a constant amount of <sup>125</sup>I-poly C9, identical dose-response inhibition curves were obtained (Fig. 5 *B*). Although there was no inhibition of this RIA with 50  $\mu$ l of NHS (bound/free ratio of 1.7), there was inhibition by the addition of 50  $\mu$ l of human serum activated with LPS (a bound/free ratio of 0.40).



Amount of Poly C9 (X) or MC5b-9 (0) (µl)

FIGURE 5 (A) RIA of Poly C9-MA: A dose-response relationship was observed for <sup>125</sup>I-poly C9 but not for increasing amounts of <sup>125</sup>I-monomeric C9. In this assay, the concentrations of C9 and poly C9 were 1.0  $\mu$ g/ml. (B) Increasing amounts of either unlabeled MC5b-9 or poly C9 (protein concentrations of 100  $\mu$ g/ml) inhibited the binding of a constant amount of <sup>125</sup>I-poly C9 in an identical manner. Addition of normal human serum (50  $\mu$ l) did not inhibit binding (bound/free = 1.7).

Immunofluorescence studies. Poly C9-MA binding was initially recognized on the internal elastic lamina and media of vessels and focally in the juxtaglomerular zone and mesangial stalk region of normal adult kidney sections (Fig. 6). There was no detectable immunofluorescence with normal fetal kidney; with other regions of mature human kidney, muscle, or skin; or with isolated lymphocytes, monocytes, polymorphonuclear leukocytes, or platelets. However, immunofluorescent positive vessels were observed in normal human liver and spleen. By phase-epifluorescence microscopy, Poly C9-MA was often recognized on phase-dense material or particles in extracellular membranes. These densities did not stain with hematoxylin-eosin. Masson Trichrome, periodic acid-Schiff, Von Kossa, Prussian blue, sudan black D, or colloidal iron stains.

The distribution and intensity of Poly C9-MA and of polyclonal antisera to human C3, C5, C6, C7, C8, and C9 were assessed on kidney sections from a variety of renal diseases. In general, Poly C9-MA bound to sites that reacted with goat antisera to the individual human complement components C5, C6, C7, C8, and C9. However, the intensity of staining was different. There was uniform conjunction of Poly C9-MA and anti-C5, which stained all tissue strongly. Antisera to C6 and C7 reacted with trace intensity, whereas anti-C8 and anti-C9 were recognized with moderate intensity. When visible, these antisera localized in the same areas as Poly C9-MA staining.

When the distribution of the MAC (as interpreted by reactivity with Poly C9-MA) was compared with that of C3, the pattern found in tissue from patients with glomerulonephritis was different from that in tissue from patients with nonnephritic diseases. In MPGN (type I and II), IgA nephropathy and anaphylactoid purpura, SLE, and membranous nephropathy, there were areas of homology in the distribution of C3 and the MAC (Fig. 7). In MPGN type II, Poly C9-MA and anti-C3 encircled dense deposits in the glomerular and tubular basement membranes, and both antisera demonstrated mesangial rings typical of dense deposit material (37). In IgA nephropathy and anaphylactoid purpura, the mesangial regions contained both the MAC and C3, although the latter was also trapped along the internal aspect of the GBM. In SLE, there was a close correlation between the distribution of C3 and the MAC in the glomerular mesangium and in subendothelial GBM deposits. In membranous nephropathy, C3 and the MAC were present in epimembranous deposits (Fig. 8). However, in addition to areas of homology in these diseases, the MAC was frequently present in a coarse granular pattern (in phase dense areas) along tubular basement membranes in loci where C3 was consistently absent.

In diabetes, hypertension, obstructive uropathy, amyloid nephropathy, and congenital dysplasia, the MAC was present in greater abundance and frequently in different loci than C3. The MAC appeared in a coarse granular pattern along tubular basement membranes and Bowman's capsule in close conjunction with the phase-dense granules previously mentioned (Fig. 9). The MAC deposition in vessel walls, internal elastic lamina, juxtaglomerular and mesangial regions and in areas of sclerosis was characteristically extensive (Fig. 10). The MAC was generally not observed trapped along the internal aspect of the GBM, except in a focal granular pattern (Fig. 11). In contrast, C3 was present focally in a linear pattern along basement membranes of the glomeruli, tubules, and Bowman's capsule; in a granular pattern in peripheral and stalk regions of the mesangium; and also in arteriolar media and intima, and frequently in the lumina of vessels. There



FIGURE 6 Immunofluorescence of a normal human glomerulus stained with Poly C9-MA (A) Poly C9-MA reacted with the internal elastic lamina and media of a small vessel (arrow), and weakly in a course granular pattern in the glomerular mesangial region.  $\times$  190. (B) A phase-contrast microscopic picture of the same glomerulus. Note small arteriole (arrow).  $\times$  190.



FIGURE 7 Immunofluorescence of Poly C9-MA on glomeruli from patients with (A) MPGN (type II) demonstrating fluorescence of the GBM and the mesangium, which on higher power reveal mesangial rings; (B) SLE with mesangial deposits; (C) IgA nephropathy with mesangial fluorescence. Staining of arteriole (arrow) is similar to that seen in normal human kidney tissue. A, B, C,  $\times$  200.

was good correlation between the areas of deposition of the MAC and (a) periodic acid-Schiff-positive material seen in the diabetic mesangium, (b) C3 deposition in diabetic fibrin caps and capsular drops, (c) areas of thioflavin T staining in amyloidosis, (d) hyalinization of hypertensive glomeruli and vessel walls, and (e) sclerosis in all forms of endstage renal disease.

When Poly C9-MA was absorbed with purified poly C9, there was complete abrogation of immunofluorescent staining on normal adult and diseased diabetic kidney tissue. Absorption with normal human plasma, diabetic plasma, and cryoprecipitate had no effect on Poly C9-MA immunofluorescence.

#### DISCUSSION

Complement-induced membrane damage is effected by the MAC, a multimolecular complex composed of the five terminal complement components. After cleavage of C5 into C5a and C5b, C6 and C7 attach to C5b, forming the C5b67 complex, which interacts with hydrophobic regions of lipid bilayers. The addition of C8 and C9 enhances the interaction of the complex with the membrane interior (5, 6, 39). In the process of MAC assembly, the terminal components undergo conformational changes, creating binding sites for phospholipids that are released from and



FIGURE 8 Immunofluorescence of a glomerulus in a patient with membranous glomerulopathy. (A) Poly C9-MA stained in a granular pattern along the GBM.  $\times$  220. Anti-C5 antibody stained with a similar intensity and distribution. (B) Staining with polyclonal anti-human C3 antibody  $\times$  220. (C) There was minimal staining with polyclonal anti-C9; reactivity with anti-C8 was equivalent in intensity and distribution to anti-C9. Anti-C6 and anti-C7 reacted with only trace intensity.  $\times$  220.



FIGURE 9 Immunofluorescence of Poly C9-MA in diabetic nephropathy (A) Extensive granular staining of Poly C9-MA in the mesangium, Bowman's capsule, afferent and efferent arterioles, and tubular basement membranes.  $\times$  280. (B) While Poly C9-MA staining was present on the inner and outer laminations of diabetic tubular basement membranes, polyclonal antibody to human C3 was absent from the inner ring (see arrow), where Poly C9-MA was present in a coarse granular pattern.  $\times$  630. (C) Localized immunofluorescence of Poly C9-MA on diabetic tubular basement membrane (arrows) corresponding to phase-dense material seen by phase-contrast microscopy of the same section (D). C and D,  $\times$  775.

which cause the reorganization of membrane lipid bilayers (39). The end result of MC5b-9 attachment is membrane damage, although the precise mechanism whereby the MC5b-9 complex disrupts the membrane barrier is uncertain. A transmembrane lipid channel may be formed because of lipid bilayer reorganization (4, 6), or, alternatively, the proteins of the MC5b-9 complex (40, 41) or poly C9 (8) alone may form a transmembrane channel.

Prior studies have shown that the assembly of the C5b-9 complex leads to the appearance of neoantigens associated with C5b6, C5b67 (7), and poly C9 (9).

This study has characterized a monoclonal antibody, Poly C9-MA, which recognizes a neoantigen of the C9



FIGURE 10 Immunofluorescent staining with Poly C9-MA was observed in vessels of kidney tissue obtained from a variety of nonnephritic patients. Tissue from a patient with hypertensive nephrosclerosis demonstrating intense immunofluorescence of the glomerulus and afferent-efferent arterioles  $(A, \times 200)$  and the media and internal elastic lamina of a larger artery  $(B, \times 410)$ . (C) An artery from a patient with amyloidosis; while there was little homology with antisera to C3 in this vessel, there was a close correlation with thioflavin T staining.  $\times 200$ .

portion of the C5b-9 complex. Evidence that the antigen reacting with Poly C9-MA is not detected in NHS but requires complement activation is derived from a number of observations. First, it was not possible to inhibit immunofluorescent reactivity of Poly C9-MA on normal human and diseased kidney sections by absorbing the antibody with normal human serum or plasma. Second, there was a lack of binding of Poly C9-MA to NHS on ELISA, but positive reactivity with complement-activated serum. Third, Poly C9-MA immunofluorescence of zymosan particles preincubated with NHS was abrogated by heat inactivation or divalent cation chelation of serum. Fourth, Poly C9-MA failed to bind to purified complement components on ELISA, and to monomeric native C9 in a sensitive RIA. Finally, human serum inhibited the <sup>125</sup>I-poly C9 RIA only after complement was activated by LPS.

That Poly C9-MA recognizes a neoantigen of C9 and not other portions of the MAC was demonstrated in two different assay systems. The zymosan immunofluorescence assay demonstrates the absence of Poly C9-MA binding to zymosan particles incubated in sera deficient in individual terminal components, and the restoration of fluorescence by reconstitution of C9-deficient sera. These results were corroborated by Poly C9-MA binding on ELISA to EAC membranes that included C5b-9, but not to intermediate complement complexes. The binding to purified polymerized C9 demonstrated by ELISA and in the RIA without reactivity to monomeric C9 documents the specificity of



FIGURE 11 Immunofluorescence of a normal human glomerulus demonstrating the contrast between (A) Poly C9-MA staining of an afferent arteriole and focally within the mesangium and (B) anti-C3 antisera staining of the same afferent arteriole and mesangium but also present along the inner aspect of the GBM. Polyclonal anti-C9 antisera stains this glomerulus in a pattern identical to that of Poly C9-MA.

Poly C9-MA. There is compelling evidence that the antigenic determinant recognized by Poly C9-MA is common to both MC5b-9 and poly C9: There was identical dose-response inhibition of the <sup>125</sup>I-poly C9 RIA by unlabeled poly C9 and by purified MC5b-9, the slopes of the MC5b-9 and poly C9 dose-response relationship on ELISA were similar, there was inhibition on ELISA of poly C9 reactivity by MC5b-9 absorption of the antibody, and there was elimination of Poly C9-MA staining on normal and diseased human kidney by absorption with poly C9.

The development of a monoclonal antibody to the MAC from collagenase digested GBM may appear fortuitous. However, the GBM preparation is only partially purified and is known to consist of a variety of renal extracellular matrix and plasma antigenic determinants (42, 43). That Poly C9-MA did not react with the preparation used for immunization reflects the scarcity of the antigen in this preparation, as well as the capacity to develop monoclonal antibodies to trace amounts of an antigen. Although the possibility that Poly C9-MA reacts with an epitope on an unrelated extracellular matrix protein is difficult to exclude, this likelihood is diminished by the lack of reaction on ELISA to known collagens or constituents of renal extracellular matrices (collagen types I, III, IV, V, fibronectin, and laminin), and the negative immunofluorescence of normal fetal kidney, human muscle and skin, and extracellular basement membranes of the adult human kidney.

Recently, Biesecker et al. (13) have demonstrated by immunofluorescence that a polyclonal antiserum to MAC binds to deposits in the kidney of patients with SLE nephritis. Although this antiserum recognizes a neoantigen(s), its specificity has not been characterized. A similar distribution of IgG, C1q, C4, and MAC was noted in glomeruli and blood vessels, whereas MAC was more frequently present in tubular deposits. Similar results were observed for the distribution of Poly C9-MA in various forms of glomerulonephritis. There was a close correlation in these diseases between the deposition C3 and that of the MAC in glomerular and mesangial regions. However, Poly C9-MA stained loci in tubular basement membranes that did not react with anti-C3, a finding which corroborates the observation of Biesecker et al. (13) in SLE.

In nonnephritic renal diseases, the MAC was present extensively in the mesangial and juxtaglomerular regions of glomeruli, internal elastic lamina and media of blood vessels, and tubular basement membranes. The deposition of the MAC was especially evident in tissues with sclerosis. In all of these loci, particularly in tubular basement membranes, there was little correlation between the distribution of C3 and that of the MAC. Additionally, the MAC was not present in loci such as the lumen of vessels and capillaries or along the internal aspect of the GBM where plasma proteins like C3 and albumin have been found. The presence of complement components in renal tissue from diseases such as hypertension, diabetes mellitus, obstruction, congenital dysplasia, and amyloid has been documented. In an immunohistochemical study of endstage kidney disease, it has been noted previously that immunoglobulins and classical and alternative pathway complement components were present in hyalinizing glomeruli, regardless of the cause of renal failure (36). Verroust et al. (44) found that multiple complement components were detected by immunofluorescence in immunoglobulin-mediated glomerular injury. Even in instances where immunoglobulin was absent, alternative pathway complement components were present. The presence of the MAC in diseased tissue is more indicative of complement activation than demonstration of individual complement components, which may reflect nonspecific trapping, as previously suggested. The deposition of the MAC in small arterioles of normal adult kidney tissue and its absence in fetal specimens is difficult to explain, but may indicate low-grade complement activation in these sites.

Whether the membrane attack complex is damaging renal extracellular matrices in a manner analogous to cell membrane disruption is uncertain, but the extensive presence of the MAC in all diseased renal tissue, especially in areas of sclerosis, suggests that complement activation may play a role in sclerosis of endstage renal disease.

The frequent disparity in the distribution between the MAC and C3 in nonnephritic diseases was consistently observed. The reason for this difference is obscure, but may be explained in a number of ways. First, C3 may be present in a form not identified by polyclonal anti-C3 antiserum. Secondly, the initiation of attack complex activation may occur at a site distant from the MAC deposition, analogous to the observations of Gotze and Müller-Eberhard (45) who have shown that the C423 enzyme can affect the binding of C5, C6, and C7 to cell surfaces physically separated from where the C423 enzyme was bound. Thirdly, Polley and Nachman (46) have described C5b-9 activation by an interaction of platelet membranes, thrombin, and C3b that appears to be independent of the classical or alternative activation pathways. Although C3b is necessary for platelet-induced C5b-9 formation, its uptake on platelet membranes was less than that observed for C8. A similar phenomenon may occur on diseased renal basement membranes. Finally, C5b-9 formation or C9 polymerization may be induced by a mechanism not previously described and not requiring binding of C3. In this regard, it is of interest that a disparity also exists between the extensive deposition of the MAC and the limited localization of immunoglobulin, especially in tissue from patients with renal disease other than glomerulonephritis.

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