The Journal of Immunology

RESEARCH ARTICLE | MAY 01 1998

Blockade of Antibody-Induced Glomerulonephritis with Crry-Ig, a Soluble Murine Complement Inhibitor[1] FREE

Richard J. Quigg; ... et. al

J Immunol (1998) 160 (9): 4553–4560.
https://doi.org/10.4049/jimmunol.160.9.4553

Blockade of Antibody-Induced Glomerulonephritis with Crry-Ig, a Soluble Murine Complement Inhibitor[1]

Richard J. Quigg,* Yuko Kozono,† Dawn Berthiaume,† Alice Lim,* David J. Salant,‡ Andrew Weinfeld,† Patricia Griffin,† Elisabeth Kremmer,§ and V. Michael Holers[2†]

A recombinant soluble form of the mouse membrane complement inhibitor Crry (complement receptor-related gene y) fused to IgG1 hinge, CH2, and CH3 domains has been created and designated Crry-Ig. Crry has been used because, similar to human soluble CR1, it demonstrates decay-accelerating activity for both the classical and alternative pathways of complement as well as cofactor activity for factor I-mediated cleavage of C3b and C4b. The mouse IgG1 isotype was incorporated because it is a noncomplement-activating isotype and, when fused to Crry, results in a complement inhibitor that should not be recognized as foreign when used chronically in murine models. Crry-Ig demonstrated complement-inhibitory activity in both the fluid phase and on target surfaces. Following in vivo injection, Crry-Ig manifested a two-phase serum elimination profile, a rapid initial loss most likely reflecting tissue redistribution and a second more prolonged decline with a $t_{1/2}$ of 40 h. Inhibition of complement activation in mice following injection of Crry-Ig was demonstrated by a marked decrease in the ability of serum from treated mice to be activated by zymosan particles in vitro. Finally, in vivo efficacy of Crry-Ig was demonstrated by its ability to substantially diminish renal injury induced by complement-fixing nephrotoxic Ab. The use of Crry-Ig in vivo in murine models of chronic inflammatory and autoimmune disease should allow further insight into the potential therapeutic effects and possible untoward complications of continuous blockade of complement using inhibitors that act on activation products of C4 and C3. ***The Journal of Immunology,*** **1998, 160: 4553–4560.**

Nephrotoxic serum nephritis is a form of Ab-mediated glomerulonephritis (GN)[3] that is readily induced in a variety of species through the passive administration of nephrotoxic serum (NTS). Binding of Ab to glomeruli in vivo is rapid, and the resultant inflammation occurs within minutes of Ab injection. In both rats and rabbits, complement activation clearly plays a pathogenic role, as complement depletion with cobra venom factor (CVF) prevents or ameliorates disease expression (1, 2). There is an apparent dose dependence, however, such that the requirement for complement is lost at higher Ab doses (1). Such complement dependence is also apparent in mice when low doses of Ab are used (3), which has been confirmed using C3- and C4-deficient mice (4).

Complement inhibitors are currently being developed and tested as potential therapeutic agents for human inflammatory, ischemic, and autoimmune diseases (reviewed in Refs. 5 and 6). In humans, soluble recombinant complement receptor type 1 (sCR1/CD35) has been developed as an inhibitor that can be used in vivo and has demonstrated therapeutic effects in well-established models of acute ischemia and inflammation (7). CR1 is a particularly potent inhibitor because it interacts with both C3 and C4, exhibits decay acceleration and factor I cofactor activity, and blocks the classical and alternative pathways (5, 8). sCR1 has been shown to decrease inflammatory endpoints in experimental models of local immune complex injury (reverse passive Arthus) (9), ischemia-reperfusion (7, 10), thermal injury (11), CVF-induced pulmonary damage (11), an Ab-mediated demyelinating form of experimental allergic encephalomyelitis (12), hyperacute xenotransplant rejection (13), and several forms of acute GN in the rat (14). A form of sCR1 has been developed that has placed the active domain of CR1 at the amino terminus of an intact Ig molecule (15). This form was predicted to have a longer $t_{1/2}$ than monomeric sCR1 as well as to potentially allow in vivo targeting using the specificity of the Ig variable domains.

sCR1 has been studied primarily in rat models for several reasons. First, previous studies using CVF have demonstrated a prominent complement-dependent component to these models. Second, many models utilize surgical techniques that have been perfected using rat models. And third, sCR1 is a particularly effective inhibitor of the rat complement cascade. In contrast, sCR1 exhibits activity only against the mouse alternative pathway and is inactive toward the classical pathway (16).

An additional limitation to the use of sCR1 is the development of a neutralizing Ab response when injected into rats or other nonhuman species. To study the effects of long-term complement inhibition, a homologous protein must be utilized in a form that is nonimmunogenic. One approach to this problem that has been developed is a homologous mouse anti-mouse C5 mAb that blocks the complement pathway at the C5 activation step (17). This mAb

*Department of Medicine, Section of Nephrology, The University of Chicago, Chicago, IL 60637; †Departments of Medicine and Immunology, Division of Rheumatology, University of Colorado Health Sciences Center, Denver, CO 80262; ‡Department of Medicine, Renal Section, Boston University Medical Center, Boston, MA 02118; and §GSF-National Research Center for Environment and Health, Institute of Immunology, Munich, Germany

Received for publication October 1, 1997. Accepted for publication January 5, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These studies were supported by National Institutes of Health Grants AI31105 (V.M.H.), DK41873 (R.J.Q.), and DK30932 (D.J.S.); a Biomedical Sciences Grant from National Arthritis Foundation (V.M.H.); and a chapter grant from Arthritis Foundation, Greater Chicago Chapter (R.J.Q.).

[2] Address correspondence and reprint requests to Dr. V. Michael Holers, Division of Rheumatology, Box B-115, University of Colorado Health Sciences Center, Denver, CO 80262. E-mail address: michael.holers@uchsc.edu

[3] Abbreviations used in this paper: GN, glomerulonephritis; Crry, complement receptor-related gene y; CVF, cobra venom factor; FPLC, fast protein liquid chromatography; IF, immunofluorescence; NTS, nephrotoxic serum; s, soluble; SLE, systemic lupus erythematosus.

0022-1767/98/$02.00

has been shown to be effective in both collagen-induced arthritis and systemic lupus erythematosus (SLE) models (18, 19).

The use of anti-C5 mAb, however, still allows the generation of active C3 and C4 cleavage fragments in vivo. To develop a mouse homologue of human sCR1 that acts on C3b and C4b, we have previously studied the enzymatic activities and complement-inhibitory capacities of the two genetic homologues of CR1, mouse CR1 and Crry (20–23). Mouse CR1 is an alternatively spliced product of the *Cr2* gene (24, 25) that acts as a membrane-bound B cell and follicular dendritic cell receptor for C3b (21, 26). Crry is a widely distributed membrane-bound intrinsic complement-regulatory protein (27, 28). In a direct comparison of CR1 and Crry, Crry was found to be a more potent complement inhibitor in that, while Crry demonstrated comparable cofactor activity as CR1 for the factor I-mediated cleavage of C3b, Crry alone was able to cleave mouse C4b in the presence of factor I (23). This observation, plus the demonstration that soluble Crry, like human sCR1, exhibits decay-accelerating activity for both the classical and alternative pathways, has led to the development of Crry rather than mouse CR1 as a homologous mouse complement inhibitor. In this study, we report the production and initial in vivo testing of an Ig chimeric form of Crry designated Crry-Ig.

Materials and Methods

Construction of Crry-Ig-encoding plasmid

A plasmid encoding the five short consensus repeats of the extracytoplasmic domain of Crry linked to the hinge, CH2, and CH3 domains of mouse IgG1 isotype was constructed (Fig. 1*A*). To prepare this construct, a plasmid designated p118-1 was obtained from Dr. Greg Hollis (Merck Research Laboratories, Rahway, NJ). p118-1 is a derivative of pOG45 (Stratagene, La Jolla, CA) in which the CMV promoter has been cloned upstream of the SV40 splice and polyadenylation sequence. Into this plasmid was cloned the exons encoding the hinge, CH2, and CH3 domains from the mouse IgG1 genomic clone (29) (kind gift of Dr. Hollis). Crry was first subcloned into the *Nhe*I site within the CD5 signal peptide and cleavage exon encoded in the CD51neg1 vector (30) (kind gift of Dr. Brian Seed, Harvard University, Cambridge, MA). Following demonstration of the production of functional soluble Crry-human IgG1 chimeric protein following transient transfection into COS-7 cells (data not shown), the CD5/Crry-containing portion of the plasmid along with ~100 bp upstream and downstream was PCR amplified with oligonucleotides that introduced *Bgl*II restriction endonuclease sites. The PCR product was digested with *Bgl*II (New England Biolabs, Beverly, MA) and subcloned into the *Bgl*II site of the p118-mIgG1 plasmid to make the plasmid p118-mIgG1-Crry (Fig. 1*A*). Nucleotide sequence analysis was performed across each cloning site to assure the anticipated outcome of this construction strategy.

Transfection and cloning of NS/0 cells secreting rCrry-Ig

Twenty micrograms of the p118-mIgG1-Crry plasmid encoding Crry-Ig were linearized with *Dra*III restriction endonuclease (New England Biolabs). The restricted DNA was then phenol and phenol/chloroform extracted and precipitated using ethanol. The pellet was washed with 70% ethanol, dried, and resuspended in 300 μl of 10 mM Tris-EDTA buffer containing 150 μg of herring sperm DNA (Boehringer Mannheim, Indianapolis, IN) made 1× HEPES-buffered saline. The nonsecreting hybridoma cell line NS/0 that had been cultured at a concentration of 1×10^5/ml was washed twice with HEPES-buffered saline and placed on ice. A quantity amounting to 1×10^8 cells was placed into a disposable cuvette and pulsed twice with one of three experimental capacitances (50 μfarads, 100 μfarads, 200 μfarads) at 300 V using a BTX Electroporator (BTX, San Diego, CA). Electroporated cells were placed immediately into limiting dilution in 96-well plates (Falcon, Franklin Lakes, NJ) and incubated overnight at 37°C. Cells were then placed in selection media with a final concentration of 400 μg/ml G418 (Mediatech, Herndon, VA). Cells were cultured for 2 wk until clones appeared. Crry-Ig levels were measured by ELISA (see below), and selected clones were chosen for further analysis. The clones utilized for Crry-Ig production underwent a continuous decrease of FCS levels in the media until they were stably growing in Iscove's media (Mediatech) with 1% FCS.

Purification of Crry-Ig using mAb-Sepharose and FPLC chromatography

Several means were utilized to purify Crry-Ig from tissue culture supernatants. First, anti-Crry mAb 10A2 (27) was dialyzed to carbonate buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 9.5). Cyanogen bromide-activated Sepharose 4B (Sigma, St. Louis, MO) was treated with 1 mM HCl and then washed with carbonate buffer according to the manufacturer's instructions. 10A2 was then added to the resin and incubated on a shaker overnight at 4°C. The resin was then blocked with 1 M glycine, pH 8, for 3 h at room temperature. The 10A2-Sepharose was then washed three times in alternating carbonate buffer and acetate buffer (0.1 M sodium acetate, 0.5 M NaCl, pH 4), followed by two washes with PBS. In all cases, the amount of unbound 10A2 was <10%.

To purify Crry-Ig, tissue culture supernatants were collected, filtered through 0.45-μm filters (Micron Separations, Westborough, MA), and then placed in a gravity flow over the column. Preliminary studies were performed to determine elution conditions that resulted in no loss of Crry-Ig complement-inhibitory activity (data not shown). For the experiments shown in this study, the column was then extensively washed in PBS and eluted with 0.1 M Na_2CO_3 and 0.5 M NaCl, pH 11. Individual fractions were neutralized with 0.1 M Tris, pH 6.8.

Crry-Ig was also purified in large scale by sequential column chromatography using an FPLC System (Pharmacia, Piscataway, NJ). Tissue culture supernatant was collected, filtered, concentrated using a Centriplus-10 (Amicon, Beverly, MA) 10,000 m.w. cutoff membrane, and then dialyzed into 10 mM K_2HPO_4, pH 7.4. The material was filtered and then loaded onto a 1.6 × 15-cm Q-Sepharose Fast Flow ion-exchange column (Pharmacia) and eluted with a salt gradient of 10 mM K_2HPO_4 and 1 M NaCl, pH 7.4. Eluted fractions were analyzed by SDS-PAGE and ELISA for Crry-Ig content. Peak fractions were then pooled, concentrated using a Centriplus-10, brought to 0.5 M NaCl, filtered, loaded onto a size exclusion column (HiLoad 26/60 Superdex; Pharmacia), and eluted with 10 mM K_2HPO_4 and 0.5 M NaCl, pH 7.4. Fractions were tested by SDS-PAGE and ELISA for purity, and then highly purified Crry-Ig was concentrated with a Centriplus-10 (Amicon) and finally dialyzed to 0.15 M NaCl and sterile filtered (Whatman, Fairfield, NJ). Purified Crry-Ig was aliquoted and frozen at −20°C. Preliminary studies demonstrated no loss of activity following at least one freeze-thaw cycle from −20°C or −70°C, or storage at 4°C for >30 days (data not shown). Amino-terminal sequence analysis was performed on purified Crry-Ig both in liquid phase and after transfer to Pro-Blot Membrane (Applied Biosystems, Foster City, CA).

ELISA for determination of Crry and Crry-Ig levels

Dynatech Immulon II (Dynatech, Chantilly, VA) 96-well plates were coated at 4°C overnight with 1 μg/well of IgG purified from rabbit anti-mouse Crry polyclonal Ab (27). Plates were then washed four times with PBS, 0.05% Tween-20 (Sigma) and blocked for 1 h at room temperature with PBS, 1% BSA (Sigma). The plates were washed four more times, and then samples diluted in PBS, 0.1% BSA were added for a 1-h incubation. Plates were then decanted and washed four times before the addition of pretitrated anti-Crry mAb 10A2 coupled with biotin (*N*-hydroxysuccinimidobiotin; Sigma) in PBS, 0.1% BSA. Following a 1-h incubation, the plates were washed four times again. Streptavidin-horseradish peroxidase (Sigma) diluted in PBS was then added for 30 min. Following four washes, ABTS (Boehringer Mannheim) activated with H_2O_2 was added. Plates were developed in the dark, and the OD_{480} was read on a Titertek Plus (ICN Biomedicals, Costa Mesa, CA).

Analysis of complement inhibition using zymosan activation

Inhibition of the alternative pathway was studied using a previously described method that utilizes flow cytometric analysis of C3 deposition on zymosan A particles (Sigma) (22). Briefly, 50 mg of zymosan particles in 10 ml of 0.15 M NaCl were first activated by boiling for 60 min, followed by washing twice in PBS. In each alternative pathway assay condition, 2×10^7 particles were added to reaction tubes containing a final concentration of 10 mM EGTA and 5 mM $MgCl_2$. Samples as described in the text were then added containing either 10 mM EDTA (negative control) or increasing amounts of Crry-Ig. Ten microliters of BALB/c serum as a source of complement were added, and all samples were brought to 100 μl with PBS. Samples were incubated at 37°C for 20 min, and the reaction was stopped by adding 10 mM EDTA. The particles were centrifuged, and supernatants were removed and frozen for later analysis. The particles were then washed twice with cold PBS, 1% BSA, and then incubated with FITC-conjugated goat anti-mouse C3 (Cappel, Durham, NC) for 1 h on ice. The samples were then washed twice in cold PBS, 1% BSA, resuspended in PBS with

1% paraformaldehyde, and then analyzed by flow cytometry using an EP-ICS cytometer (Coulter, Hialeah, FL). Percentage inhibition was calculated using the formula [1 − [sample mean channel fluorescence − background (10 mM EDTA condition)/positive control mean channel fluorescence (no Crry-Ig) − background]] × 100.

Supernatants from the reaction were also analyzed by Western blotting to determine the extent of C3 cleavage. In this analysis, 5 μl of the supernatant was mixed with an equal amount of SDS-PAGE loading buffer with 10% 2-ME. The samples were subjected to SDS-PAGE on a 7.5% acrylamide gel, transferred to Hybond enhanced chemoluminescence (ECL) paper (Amersham, Arlington Heights, IL) overnight in 0.19 M Tris, 0.025 M glycine, 20% methanol buffer. Following this, membranes were blocked in PBS, 0.1% Tween with 10% milk for 1 h. Anti-C3 mAb RmC11H9 (31) that had been pretitered was then added to the blot in the same buffer with 1% BSA. Following washing, horseradish peroxidase-conjugated goat anti-rat IgG (Southern Biotechnology, Birmingham, AL) (preadsorbed against mouse IgG) was added for 1 h, and then the blot was washed and developed using the enhanced chemoluminescence (ECL) system (Amersham).

In vivo kinetics of Crry-Ig

BALB/c mice were injected with either 1 or 3 mg of Crry-Ig in sterile 0.15 M NaCl by either i.p. or i.v. routes. Blood was collected before Crry-Ig injection and then at time points as indicated in *Results*. For collection of serum with active complement, blood was allowed to clot at room temperature for 10 min and then on ice for 15 min. The samples were then centrifuged and serum stored at −70°C in aliquots for later analysis.

Induction of nephrotoxic nephritis and inhibition with Crry-Ig and CVF

NTS was raised by immunizing a single sheep with rat glomeruli in CFA, and the complement-fixing IgG1 subclass was isolated by anion-exchange chromatography on DEAE-Sephacel (32). This antiserum cross-reacts with mouse glomeruli, to which it binds in vivo in a linear peripheral capillary pattern (33). NTS was injected via the tail vein into 25 to 30 g female CD-1 mice (Harlan Sprague-Dawley, Indianapolis, IN).

In initial studies, NTS IgG was injected into complement-sufficient mice and those in which complement was either depleted with CVF (*Naja naja kaouthia*; Quidel, San Diego, CA) or inhibited with Crry-Ig. Twelve units of CVF in 0.5 ml PBS were administered 24 and 16 h before disease induction, while the other two groups received PBS alone. Animals were injected i.v. with 0.5 mg of NTS IgG. Crry-Ig-treated animals received 1.5 mg of Crry-Ig i.v. at the same time as NTS IgG, while the other two groups received buffer such that the total volume injected i.v. was 0.2 ml in all animals. Two hours after disease induction, animals were anesthetized, blood was collected by cardiac puncture, urine was collected with a Pasteur pipette from the urethra, while the bladder was gently compressed and renal tissue was harvested.

The effects of Crry-Ig given before injection of NTS were also evaluated. Animals were injected i.p. with 1.5 mg of Crry-Ig or buffer alone. Two hours later, animals received either 0.5 or 2 mg of NTS IgG i.v. Animals were housed in metabolic cages for 18 h, during which time urine was collected. Mice were then sacrificed to harvest blood and renal tissue.

Urinary albumin concentration was measured by ELISA, as previously described (34), while urinary creatinine was measured with a Beckman Autoanalyzer. To normalize albumin excretion, data are expressed as micrograms of albumin per milligram of creatinine (34, 35). Normal mice excrete less than 25 μg of albumin/mg of creatinine, similar to the case in humans. Crry-Ig was measured by ELISA in both sera and urine. Mouse C3 was measured in sera by ELISA, as previously described (36). Direct immunofluorescence (IF) microscopy for sheep IgG and mouse C3 was performed on snap-frozen renal tissue (37). Renal tissue was also subjected to immunohistochemical staining with mAb 7/4 for mouse neutrophils (Serotec, Oxford, U.K.). The number of positively stained cells was counted in at least 60 glomeruli per animal (34). Glomerular IF scoring and neutrophil counts were accumulated in a blinded fashion.

Statistics

Statistical analyses were performed with Minitab software (College Park, PA). Data are expressed as mean ± SEM. When more than two groups were evaluated, comparisons were made by one-way analyses of variance. Urinary albumin excretion was compared between Crry-Ig and control-treated groups by Mann-Whitney testing. IF staining between groups was compared by χ^2 analysis.

Results

Production, purification, and characterization of Crry-Ig

Crry was chosen as a recombinant complement inhibitor that could be used chronically as a homologous therapeutic agent. Previously, a soluble form of monomeric Crry was created and produced in baculovirus (22, 27). This form was shown to retain decay-accelerating activity for both the classical and alternative pathways as well as cofactor activity for factor I-mediated cleavage of both C3 and C4. Because many previous studies have demonstrated that recombinant molecules have substantially longer $t_{1/2}$ when fused to Ig Fc-containing domains (38, 39), an approach that incorporated the generation of an Ig chimeric protein containing Crry was pursued.

Figure 1*A* demonstrates the plasmid that was constructed and includes the important cloning sites and relative positions of the nucleotides encoding the signal peptide, Crry and IgG1 hinge, and CH2 and CH3 domains. Not shown is the inclusion of a neomycin-resistance cassette derived from the original pOG45 plasmid. Figure 1*B* illustrates the structure of Crry-Ig that is directed by the plasmid p118-IgG1-Crry. Relevant features of the recombinant protein include the retention of the Fc portion of the non-complement-fixing mouse IgG1 isotype and the lack of light chains derived from either the plasmid or the NS/0 cell line.

Following transfection of NS/0 cells, cloning by limiting dilution, and G418 selection, one clone was chosen for further analysis based on stable growth characteristics and levels of protein production. This clone routinely accumulates 50 mg/L of supernatant when grown in 1% FCS-containing media. Initial studies utilized protein purified using mAb affinity chromatography (Fig. 2, *lane 3*), although later studies with Crry-Ig purified by a high capacity two-column FPLC method (Fig. 2, *lane 4*) demonstrated identical results. The highly purified band of 160 kDa in *lanes 3* and *4* specifically reacted in Western blots with both the anti-Crry mAb 10A2 as well as polyclonal anti-Crry Ab (data not shown). Purified Crry-Ig was also subjected to amino-terminal sequence analysis to assure proper signal peptide cleavage. The results demonstrated the anticipated sequence DHCPAPSQLPSAKPINLT, which is authentic Crry (40) with the addition of the amino-terminal D derived from the CD5 signal peptide cleavage site.

Crry-Ig blocks complement activation in whole serum

Experiments were performed to measure the complement-inhibitory activity of Crry-Ig using a previously published zymosan activation assay (22). In this assay, zymosan was added to 10% mouse serum in the presence of Mg^{2+}/EGTA, and the level of alternative pathway activation was assessed. Inhibition of complement activation is manifest by a decrease in the amount of C3 bound to the surface of the zymosan particles when assessed using flow cytometry. Figure 3*A* demonstrates the percentage inhibition of C3 deposition at increasing doses of Crry-Ig. At a level of 10 to 12 μg/ml of Crry-Ig, essentially complete inhibition of C3 deposition was found. When comparing the activity of Crry-Ig to monomeric Crry produced in baculovirus (22), equal inhibition of activity on a Crry molar-equivalent basis was found (data not shown).

Although these results demonstrated a decrease in C3 deposition, we sought to confirm that Crry-Ig also blocked C3 activation in the fluid phase. Therefore, supernatants from the zymosan reaction were also analyzed by Western blotting using a mAb that detects both the intact C3 α and cleaved α'-chains (31). As shown in Figure 3*B*, C3 α-chain disappeared in the fluid phase coincident with its appearance on zymosan, as assessed by flow cytometry. At doses that completely blocked C3 deposition onto the zymosan

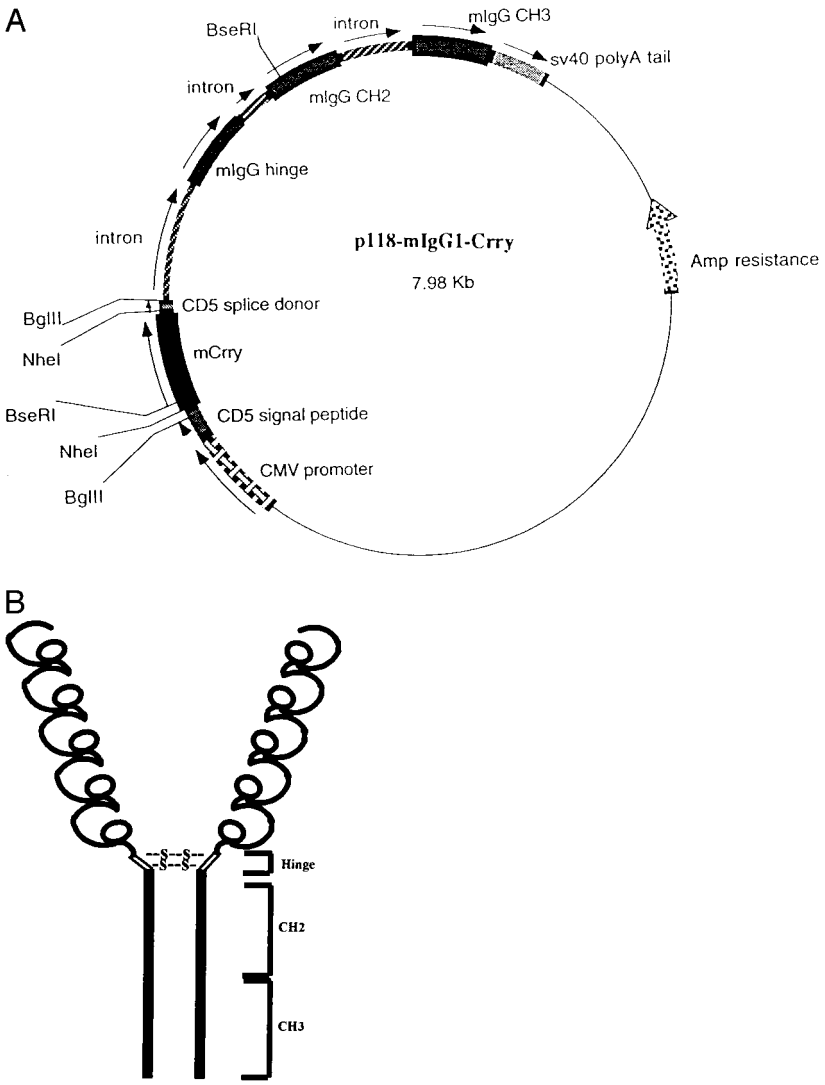

FIGURE 1. *A*, Schematic representation of the plasmid encoding the CD5 signal peptide and the five extracytoplasmic short consensus repeats of Crry, followed by the hinge, CH2, and CH3 domains. *B*, Schematic diagram of the predicted Crry-Ig homodimer encoded by the plasmid shown in *A*.

particles, no α' fragments were detected, and the level of intact C3 α-chain was identical to the EDTA control. Therefore, in addition to confirming that the decreased C3 deposition on zymosan is due to blockade of complement activation, these results demonstrate that soluble Crry-Ig has the capacity to block complement activation in the fluid phase as well as on target surfaces. This observation supports the use of Crry-Ig as an inhibitor in vivo in settings in which complement is activated in many tissue sites on both targets and in the fluid phase.

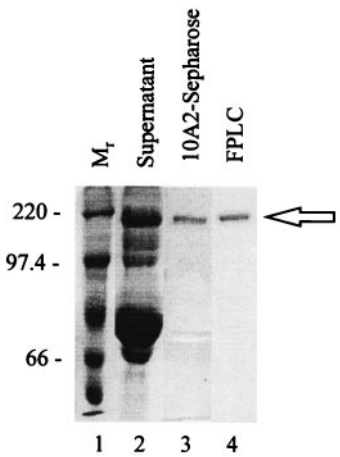

FIGURE 2. Coomassie blue stain of a 7.5% polyacrylamide gel in which the supernatant of the clone producing Crry-Ig in Iscove's media with 1% FCS (*lane 2*) was compared with 1 μg of Crry-Ig purified using either mAb 10A2-Sepharose (*lane 3*) or the FPLC method (*lane 4*).

Experiments were then performed to determine the in vivo $t_{1/2}$ of Crry-Ig as well as test whether inhibition of complement activation resulted from the use of Crry-Ig. Preliminary experiments were first performed in which we confirmed the previously reported absence in the basal state of detectable serum or urine Crry using Western blot analysis (27) by using the new ELISA technique (data not shown). Following this, 1 mg of Crry-Ig was injected either i.v. (Fig. 4*A*) or i.p. (Fig. 4*B*), and levels were measured at time points up to 48 h. Crry-Ig levels rapidly rose to peak levels measured at 30 to 60 min, followed by a two-phase elimination profile. The clearance in the first phase was relatively rapid, most likely reflecting tissue redistribution. This was followed by a more prolonged second phase with a $t_{1/2}$ of 40 h (measured from the i.v. injection profile). A rapid initial elimination followed by a prolonged second phase is similar to that previously reported for CTLA-4Ig ($t_{1/2}$ ~30 h) (41) as well as IFN-γ receptor Ig fusion proteins ($t_{1/2}$ 44 h) (39). Thus, Crry-Ig acts in vivo in the predicted fashion and comparable with other biologics that have been fused to Ig domains using recombinant techniques.

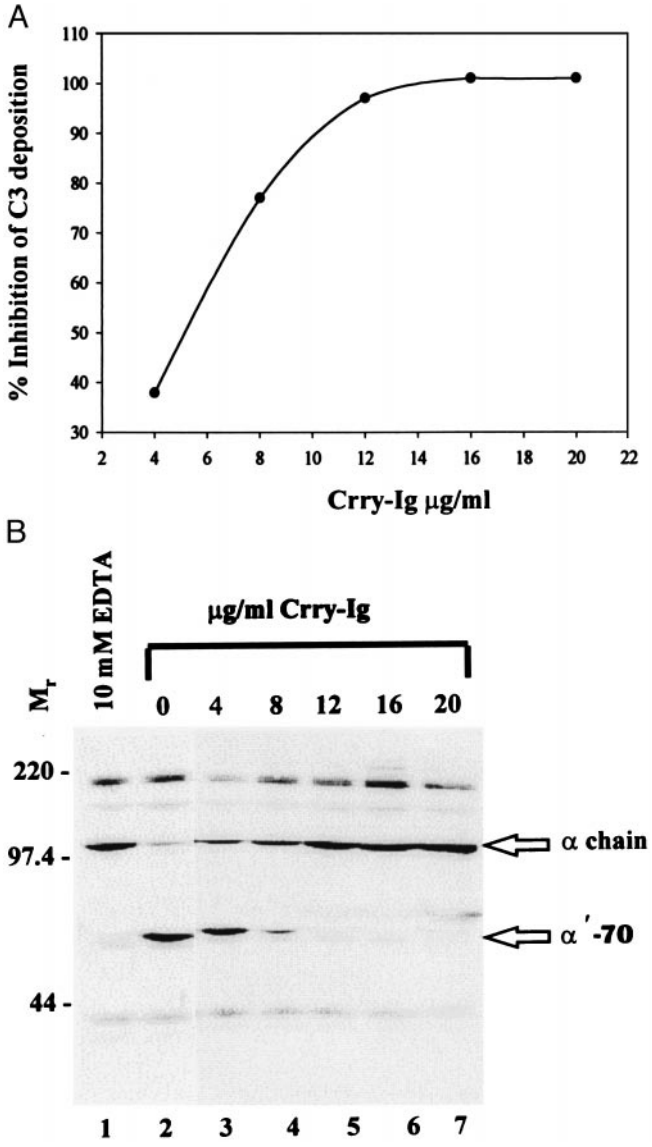

FIGURE 3. Representative experiment in which the inhibition of complement activation by Crry-Ig on zymosan particles was analyzed following flow cytometry to detect surface-bound C3 (*A*), or when an aliquot of the zymosan reaction supernatant was analyzed by Western blotting and detection using anti-C3 mAb (*B*). Positions of the intact C3α and C3α'-70 chains in *B* are shown by arrows at *right*. The 10 mM EDTA lane represents the negative control, and increasing doses of Crry-Ig are shown at the *top* in *lanes 2* to *7*.

The ability of Crry-Ig to block complement activation in sera following in vivo injection was then assessed. Sera from mice in which either 1 or 3 mg Crry-Ig had been injected i.p. 1 h prior were studied. Mean serum levels of Crry-Ig following 1 mg injection were 49.3 $\pm$ 4.9 μg/ml ($n = 4$), and following 3 mg injection were 232.5 $\pm$ 35.4 μg/ml ($n = 3$). Inhibition of complement activation was apparent, as the ability of zymosan to activate complement in vitro was inhibited by 79.3% $\pm$ 5.2% and 94.7 $\pm$ 2.7% for the 1 and 3 mg doses, respectively. Since in this assay whole serum was diluted 10-fold, the resulting Crry-Ig levels and degree of complement inhibition are comparable with those in which Crry-Ig was directly added to 10% normal serum in the experiment depicted in Figure 3. Therefore, no proteins that interfere with Crry-Ig activity are present in normal BALB/c serum, and a complement-inhibitory effect of the recombinant molecule is readily demonstrable in vivo.

Effects of Crry-Ig on nephrotoxic nephritis

In initial studies of nephrotoxic nephritis, the effects of complement depletion with CVF were compared directly with those of complement inhibition with Crry-Ig. Thus, complement was either depleted with CVF given i.p. before NTS administration, or inhibited with 1.5 mg of Crry-Ig given i.v. at the time of NTS injection. At sacrifice, C3 levels were 0.40 $\pm$ 0.06 mg/ml ($n = 7$) in CVF-treated mice, compared with 2.89 $\pm$ 0.29 ($n = 5$) and 3.05 $\pm$ 0.27 mg/ml ($n = 6$) in PBS- and Crry-Ig-treated mice, respectively. As shown in Figure 5, complement depletion or inhibition led to >50% reduction in albuminuria at 2 h after injection of NTS IgG ($p = 0.072$ by analysis of variance). This degree of inhibition is similar to that seen when C3- and C4-deficient mice are injected with NTS (4). Glomerular neutrophil counts were not elevated significantly in these studies, consistent with the low dose of NTS IgG administered. For example, in the PBS- and CVF-treated groups, there were 0.15 $\pm$ 0.07 and 0.05 $\pm$ 0.01 neutrophils per glomerulus ($p > 0.05$).

Further studies were then performed with a longer observation period and with the use of Crry-Ig given i.p. Mice received either 2 mg of Crry-Ig i.p. ($n = 10$) or a comparable volume of PBS ($n = 11$), followed in 2 h by 0.5 mg of NTS IgG i.v. Animals were then placed in metabolic cages for 18 h, after which they were sacrificed. Individual data points for urinary albumin excretion are shown in Figure 6. Animals treated with PBS excreted 462.2 $\pm$ 118.9 μg albumin/mg creatinine, while animals treated with Crry-Ig had 300.1 $\pm$ 136.3 μg albumin/mg creatinine ($p < 0.05$), illustrating that complement inhibition with rCrry-Ig significantly reduced proteinuria. As shown in this figure, one animal treated with Crry-Ig had 1503.8 μg/mg creatinine, which was the highest in both groups and could not be explained upon review of available data. If this animal was removed from analysis, urinary albumin excretion was 166.3 $\pm$ 29.3 μg albumin/mg creatinine ($p < 0.01$ vs PBS-treated animals). At sacrifice, Crry-Ig-treated animals had serum Crry-Ig levels of 83.1 $\pm$ 10.7 μg/ml. These animals also had detectable urinary Crry-Ig (3.8 $\pm$ 0.5 μg Crry-Ig/mg creatinine), which was positively correlated with serum Crry-Ig concentration ($p < 0.05$). The fractional excretion of Crry-Ig compared with creatinine, which freely crosses the glomerulus, was 0.013%, indicating limited passage of Crry-Ig across the glomerular capillary wall.

By IF microscopy, all animals had intense linear staining for sheep IgG that was indistinguishable between the two groups (Fig. 7), suggesting that Crry-Ig did not affect glomerular binding of the NTS IgG. In 4 of 11 PBS-treated animals, there was discontinuous glomerular capillary IF staining for C3 (Fig. 8*A*), while C3 staining was absent in all Crry-Ig-treated animals (Fig. 8*B*) ($p < 0.05$ by χ^2 analysis).

To determine whether inhibition of injury in nephrotoxic nephritis is dependent upon the dose of NTS IgG used, additional studies were performed in which animals received 2 mg of NTS IgG. There was no significant difference between Crry-Ig-treated animals and controls with nephrotoxic nephritis, with respective urinary albumin excretions of 7,826.2 $\pm$ 1,346.3 and 13,284.7 $\pm$ 3,583.5 μg albumin/mg creatinine ($n = 4$ each). Thus, the apparent complement dependence of nephrotoxic nephritis is eliminated at higher doses of NTS, which is a well-known phenomenon (1, 3).

Discussion

These studies have demonstrated the utility of using Crry-Ig, a mouse functional analogue of human sCR1, as an inhibitor in an in vivo complement-dependent model of acute inflammation. Using a strategy that incorporates the addition of a non-complement-fixing

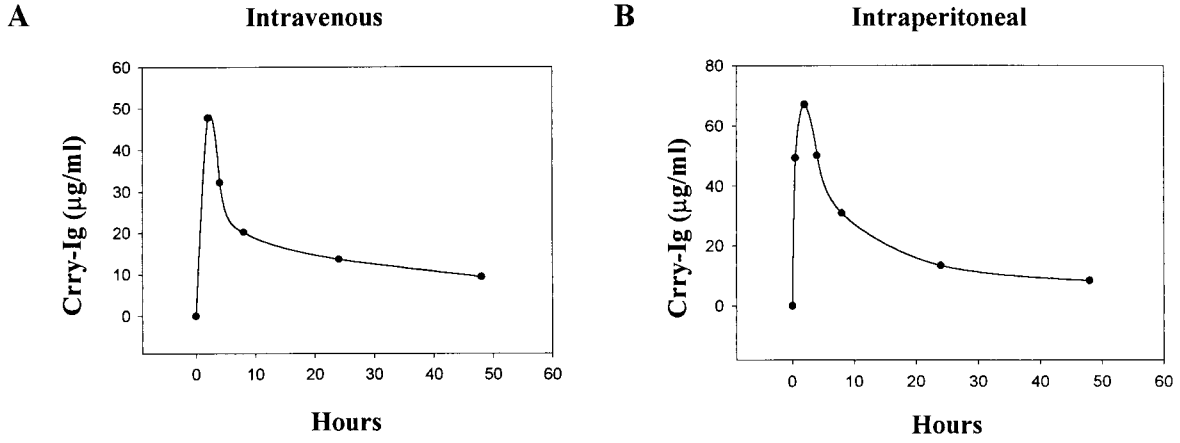

FIGURE 4. Mean serum levels of Crry-Ig measured by ELISA following injection of 1 mg i.v. (*A*) or i.p. (*B*) into two mice each. An initial rapid phase of elimination is followed by a more prolonged elimination with a $t_{1/2}$ of 40 h.

mouse IgG1 Fc region to the functional domain of mouse Crry, a molecule with potent in vivo inhibitory activity targeted at C4 and C3 is now available for studies in mouse models of acute and chronic inflammatory and autoimmune diseases. Based on the similarity of specific enzymatic activities shared by human sCR1 and Crry, results obtained using Crry-Ig chronically should be considered as comparable with those that would be expected from the use of a similar form of sCR1 in chronic human diseases.

As a model of glomerular disease, nephrotoxic nephritis (also known as experimental antiglomerular basement membrane GN) has been studied widely in various forms and has provided insights into the complex immunologic events that occur in GN. Recently, a number of inhibitory compounds have been used in this model, further helping to elucidate pathogenic mechanisms. In the acute heterologous phase in rats, the use of IL-1R antagonists (42), anti-TNF-α mAb (43), Ab to several adhesion molecules, such as ICAM-1 and CD11b (43, 44), anti-chemokine Abs (45, 46), and defibrinogenation or blockade of platelet fibrinogen receptors (47) all partially reduce early disease manifestations (proteinuria and neutrophil infiltration). However, complete prevention of these disease manifestations in rats has been achieved by complement depletion with CVF (2).

Through the elegant studies by Boyce and Holdsworth in rabbits, it is clear that the mediator systems in so-called anti-glomerular basement membrane GN are dependent on the dose of Ab (1). As the dose is raised, the disease first becomes neutrophil independent and subsequently neutrophil and complement independent. Indeed, a component of the injury induced by the NTS used in the present studies is complement and neutrophil independent and may be due to direct reactivity with cell surface Ags on glomerular cells (48). Nevertheless, these studies have shown that a substantial fraction of the proteinuria in nephrotoxic nephritis is due to Ab-directed complement activation. Our results confirm past studies in which CVF was used (3) and recent studies with C3- and C4-deficient mice (4). Most importantly, we have extended these studies and documented that Crry-Ig is effective in reducing albuminuria to a comparable extent as CVF, validating the effectiveness of this complement inhibitor in vivo. Taken together, our results lend support to the hypothesis that Ab-directed complement activation initiates a cascade involving cytokine production, up-regulation of adhesion molecules and chemokine production, and recruitment and activation of platelets and neutrophils, which ultimately lead to this early glomerular injury (43, 45, 49).

We do recognize that nephrotoxic nephritis may not be the ideal model to document the relative effectiveness of Crry-Ig, as the complement dependence of GN is only partial in this strain. There is relatively minor glomerular C3 deposition evident by IF microscopy, and serum complement levels are normal. This is in contrast to other human disease states and experimental models of glomerular injury, such as lupus nephritis, in which intense glomerular complement deposition occurs and systemic complement consumption is apparent. However, first validating the use of Crry-Ig

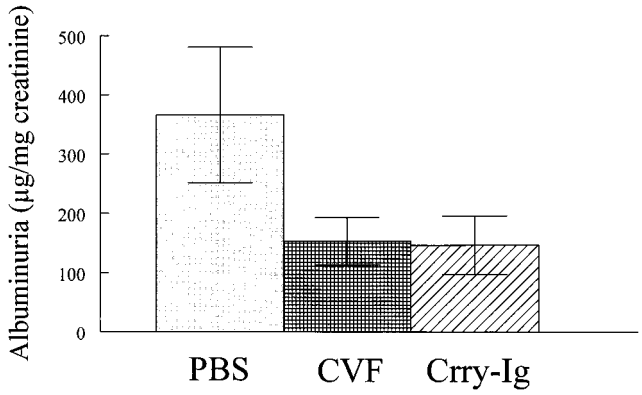

FIGURE 5. Effects of complement depletion or inhibition on NTS IgG-induced albuminuria. Complement was depleted with CVF or inhibited with Crry-Ig. Control animals were given buffer (PBS) alone. Urine was collected 2 h after NTS injection. Data are mean ± SEM.

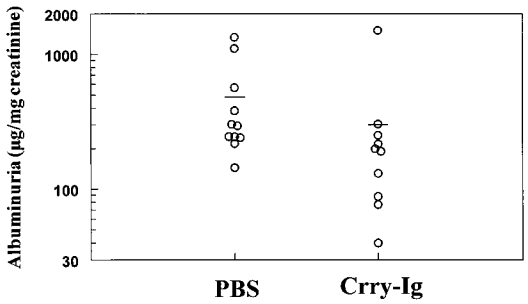

FIGURE 6. Effect of Crry-Ig on NTS IgG-induced albuminuria. Crry-Ig was given i.p. 2 h before NTS injection, following which urine was collected for 18 h. Data from individual animals are shown with the mean in each group as a horizontal line.

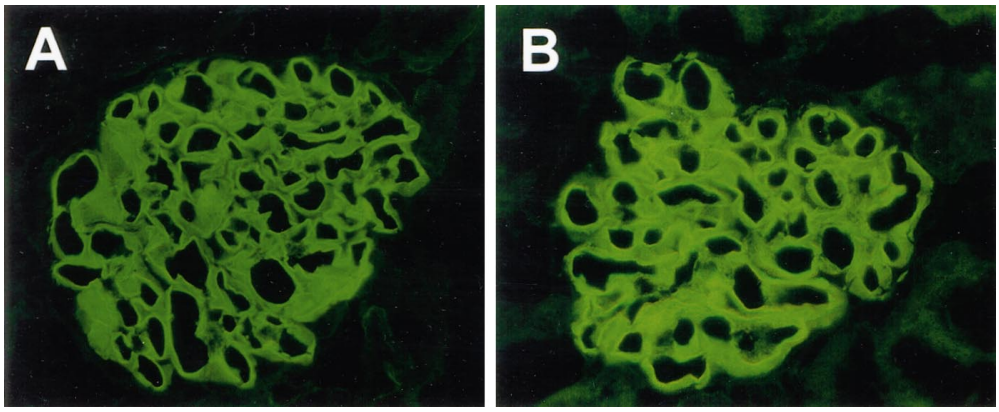

FIGURE 7. IF staining for sheep IgG in representative glomeruli from mice given sheep NTS IgG. Animals were treated with either PBS alone (*A*) or Crry-Ig (*B*).

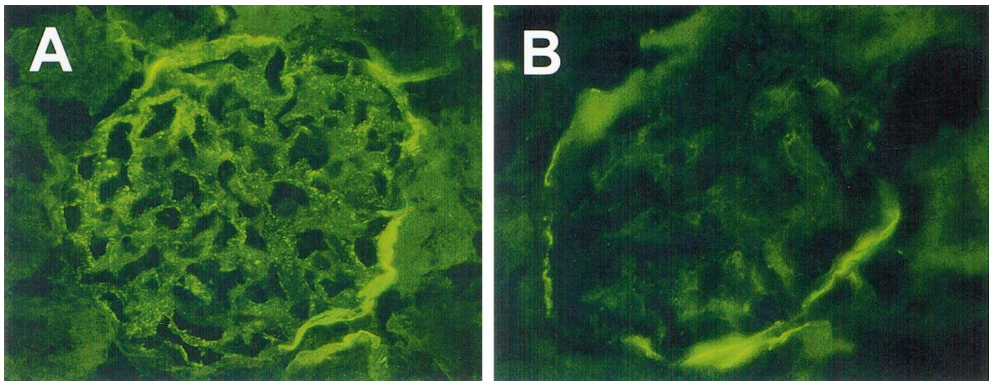

FIGURE 8. IF staining for mouse C3 in glomeruli from mice given NTS IgG. Animals were treated with either PBS alone (*A*) or Crry-Ig (*B*).

in a short-term model of GN, as we have done in this study, supports the use of Crry-Ig in the longer term models of GN, such as in spontaneous murine models of SLE.

The use of Crry-Ig in mouse models also incorporates a number of features that will allow the testing of hypotheses related to the use of complement inhibitors. First, although it is well established that complement inhibition ameliorates several manifestations of acute inflammation, it is not known whether complement inhibition will also be effective in chronic inflammatory states in which many mechanisms of tissue damage are involved. Because Crry-Ig is a homologous inhibitor with a long $t_{1/2}$, the long-term therapeutic efficacy of complement inhibition can be assessed. Second, anti-C5 mAb therapy has been reported by Wang and coworkers to be effective in mouse in the collagen-induced arthritis model (50) and the (NZB $\times$ NZW)F_1 model of SLE (19). However, unlike anti-C5 mAb therapy, Crry-Ig (and the human inhibitor sCR1) block complement activation at earlier points before the generation of large amounts of C3a and C4a activation fragments. Blockade at earlier points in the pathway may result in more (or less) therapeutic efficacy, and a strategy utilizing Crry-Ig and sCR1 chronically needs to be tested and directly compared with others. Third, Crry-Ig itself can be produced and purified in sufficiently large amounts using the NS/0 system that has been established, such that its efficacy can be studied in many different chronic models. And finally, using transgenic technology, we have created a mouse strain in which the synthesis of soluble Crry is directed by the metallothionein promoter (R. Quigg and V. M. Holers, unpublished observations). Comparison of mice treated with Crry-Ig with those that are exposed continuously to comparable levels of endogenously produced serum Crry should provide further insights into the biologic roles of complement and the risk-benefit ratios inherent in the therapeutic use of complement inhibitors.

Acknowledgments

We thank Shad Eubanks and John Freed at National Jewish Medical and Research Center Protein Facility for sequence analysis of Crry-Ig, and Dr. Joel Guthridge for invaluable assistance in protein purification.

References

1. Boyce, N. W., and S. R. Holdsworth. 1985. Anti-glomerular basement membrane antibody-induced experimental glomerulonephritis: evidence for dose-dependent direct antibody and complement-induced, cell-independent injury. *J. Immunol. 135:3918.*
2. Lefkowith, J. B., T. Nagamatsu, J. Pippin, and G. F. Schreiner. 1991. Role of leukocytes in metabolic and functional derangement of experimental glomerulonephritis. *Am. J. Physiol. Renal Fluid Electrolyte Physiol. 261:F213.*
3. Schrijver, G., K. J. M. Assmann, M. J. J. T. Bogman, J. C. M. Robbe, R. M. W. de Waal, and R. A. P. Koene. 1988. Antiglomerular basement membrane nephritis in the mouse. *Lab. Invest. 59:484.*
4. Brady, H. R., M. J. Hebert, T. Takano, A. Papayianni, H. G. Rennke, D. Sandstrom, A. W. Minto, D. J. Salant, and M. C. Carroll. 1995. Acute nephrotoxic serum nephritis in complement "knockout" mice: relative roles of the classical and alternative pathways in neutrophil recruitment and proteinuria. *J. Am. Soc. Nephrol. 6:862 (Abstr.).*
5. Kalli, K. R., P. Hsu, and D. T. Fearon. 1994. Therapeutic uses of recombinant complement protein inhibitors. *Springer Semin. Immunopathol. 15:417.*
6. Moore, F. D. 1994. Therapeutic regulation of the complement system in acute injury states. *Adv. Immunol. 56:267.*
7. Weisman, H. F., T. Bartow, M. K. Leppo, H. C. Marsh Jr., G. R. Carson, M. F. Concino, M. P. Boyle, K. H. Roux, M. L. Weisfeldt, and D. T. Fearon. 1990. Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis. *Science 249:146.*
8. Liszewski, M. K., T. C. Farries, D. M. Lublin, I. A. Rooney, and J. P. Atkinson. 1996. Control of the complement system. *Adv. Immunol. 61:201.*
9. Yeh, C. G., H. C. Marsh Jr., G. R. Carson, L. Berman, M. F. Concino, S. M. Scesney, R. E. Kuestner, R. Skibbens, K. A. Donahue, and S. H. Ip. 1991.

Recombinant soluble human complement receptor type 1 inhibits inflammation in the reversed passive arthus reaction in rats. *J. Immunol. 146:250.*
10. Hill, J., T. F. Lindsay, F. Ortiz, C. G. Yeh, H. B. Hechtman, and F. D. Moore, Jr. 1992. Soluble complement receptor type 1 ameliorates the local and remote organ injury after intestinal ischemia-reperfusion in the rat. *J. Immunol. 149:1723.*
11. Mulligan, M. S., C. G. Yeh, A. R. Rudolph, and P. A. Ward. 1992. Protective effects of soluble CR1 in complement- and neutrophil-mediated tissue injury. *J. Immunol. 148:1479.*
12. Piddlesden, S. J., M. K. Storch, M. Hibbs, A. M. Freeman, H. Lassmann, and B. P. Morgan. 1994. Soluble recombinant complement receptor 1 inhibits inflammation and demyelination in antibody-mediated demyelinating experimental allergic encephalomyelitis. *J. Immunol. 152:5477.*
13. Ryan, U. S. 1995. Complement inhibitory therapeutics. *Nat. Med. 1:967.*
14. Couser, W. G., R. J. Johnson, B. A. Young, C. G. Yeh, C. A. Toth, and A. R. Rudolph. 1995. The effects of soluble recombinant complement receptor 1 on complement-mediated experimental glomerulonephritis. *J. Am. Soc. Nephrol. 5:1888.*
15. Kalli, K. R., P. Hsu, T. J. Bartow, J. M. Ahearn, A. Matsumoto, L. B. Klickstein, and D. T. Fearon. 1991. Mapping of the C3b-binding site of CR1 and construction of a (CR1)2-F(ab′)$_2$ chimeric complement inhibitor. *J. Exp. Med. 174:1451.*
16. Pemberton, M., G. Anderson, V. Vetvicka, D. E. Justus, and G. D. Ross. 1993. Microvascular effects of complement blockade with soluble recombinant CR1 on ischemia/reperfusion injury of skeletal muscle. *J. Immunol. 150:5104.*
17. Frei, Y., J. D. Lambris, and B. Stockinger. 1987. Generation of a monoclonal antibody to mouse C5: application in an ELISA assay for detection of anti-C5 antibodies. *Mol. Cell. Probes 1:141.*
18. Wang, Y., S. A. Rollins, J. A. Madri, and L. A. Matis. 1995. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc. Natl. Acad. Sci. USA 92:8955.*
19. Wang, Y., J. A. Madri, S. A. Rollins, A. Chodera, and L. A. Matis. 1996. Amelioration of lupus-like autoimmune disease in NZB/W F_1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proc. Natl. Acad. Sci. USA 93:8563.*
20. Molina, H., W. Wong, T. Kinoshita, C. Brenner, S. Foley, and V. M. Holers. 1992. Distinct receptor and regulatory properties of recombinant mouse complement receptor 1 (CR1) and Crry, the two genetic homologs of human CR1. *J. Exp. Med. 175:121.*
21. Molina, H., T. Kinoshita, C. B. Webster, and V. M. Holers. 1994. Analysis of C3b/C3d binding sites and factor I cofactor regions within mouse complement receptors 1 and 2. *J. Immunol. 153:789.*
22. Foley, S., B. Li, M. Dehoff, H. Molina, and V. M. Holers. 1993. Mouse Crry/p65 is a regulator of the alternative pathway of complement activation. *Eur. J. Immunol. 23:1381.*
23. Kim, Y. U., T. Kinoshita, H. Molina, D. Hourcade, T. Seya, L. M. Wagner, and V. M. Holers. 1995. Mouse complement regulatory protein Crry/p65 utilizes the specific mechanisms of both decay-accelerating factor and membrane cofactor protein. *J. Exp. Med. 181:151.*
24. Kurtz, C. B., E. O'Toole, S. M. Christensen, and J. H. Weis. 1990. The murine complement receptor gene family. IV. Alternative splicing of *Cr2* gene transcripts predicts two distinct gene products that share homologous domains with both human CR2 and CR1. *J. Immunol. 144:3581.*
25. Molina, H., T. Kinoshita, K. Inoue, J.-C. Carel, and V. M. Holers. 1990. A molecular and immunochemical characterization of mouse CR2: evidence for a single gene model of mouse complement receptors 1 and 2. *J. Immunol. 145: 2974.*
26. Kinoshita, T., G. Thyphronitis, G. C. Tsokos, F. D. Finkelman, K. Hong, H. Sakai, and K. Inoue. 1990. Characterization of murine complement receptor type 2 and its immunological cross-reactivity with type 1 receptor. *Int. Immunol. 2:651.*
27. Li, B., C. Sallee, M. Dehoff, S. Foley, H. Molina, and V. M. Holers. 1993. Mouse Crry/p65: characterization of monoclonal antibodies and the tissue distribution of a functional homologue of human MCP and DAF. *J. Immunol. 151:4295.*
28. Wong, W. W., and D. T. Fearon. 1985. p65: a C3b-binding protein on murine cells that shares antigenic determinants with the human C3b receptor (CR1) and is distinct from murine C3b receptor. *J. Immunol. 134:4048.*
29. Honjo, T., M. Obata, Y. Yamawaki-Kataoka, T. Kataoka, T. Kawakami, N. Takahashi, and Y. Mano. 1979. Cloning and complete nucleotide sequence of mouse immunoglobulin gamma-1 chain gene. *Cell 18:559.*
30. Aruffo, A., I. Stamenkovic, M. Melnick, C. B. Underhill, and B. Seed. 1990. CD44 is the principal cell surface receptor for hyaluronate. *Cell 61:1303.*
31. Kremmer, E., S. Thierfelder, E. Felber, G. Hoffmann-Fezer, and M. Wasiliu. 1990. Monoclonal antibodies to complement components without the need of their prior purification. II. Antibodies to mouse C3 and C4. *Hybridoma 9:309.*
32. Lloyd, C. M., A. W. Minto, M. E. Dorf, A. Proudfoot, T. N. Wells, D. J. Salant, and J. C. Gutierrez-Ramos. 1997. RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. *J. Exp. Med. 185:1371.*
33. Salant, D. J., and A. V. Cybulsky. 1988. Experimental glomerulonephritis. *Methods Enzymol. 162:421.*
34. Quigg, R. J., A. Lim, M. Haas, J. J. Alexander, C. He, and M. C. Carroll. 1998. Immune complex glomerulonephritis in C4- and C3-deficient mice. *Kidney Int. 53:320.*
35. Mayadas, T. N., D. L. Mendrick, H. R. Brady, T. Tang, A. Papayianni, K. J. M. Assmann, D. D. Wagner, R. O. Hynes, and R. S. Cotran. 1996. Acute passive anti-glomerular basement nephritis in P-selectin-deficient mice. *Kidney Int. 49:1342.*
36. Quigg, R. J., J. J. Alexander, C. F. Lo, A. Lim, C. He, and V. M. Holers. 1997. Characterization of C3-binding proteins on mouse neutrophils and platelets. *J. Immunol. 159:2438.*
37. Quigg, R. J., D. R. Abramson, A. V. Cybulsky, J. Badalamenti, A. W. M. Minto, and D. J. Salant. 1989. Studies with antibodies to cultured rat glomerular epithelial cells: subepithelial immune deposit formation after in vivo injection. *Am. J. Pathol. 134:1125.*
38. Capon, D. J., S. M. Chamow, J. Mordenti, S. A. Marsters, T. Gregory, H. Mitsuya, R. A. Byrn, C. Lucas, F. M. Wurm, J. E. Groopman, S. Broder, and D. H. Smith. 1989. Designing CD4 immunoadhesins for AIDS therapy. *Science 337:525.*
39. Kurschner, C., L. Ozmen, G. Garotta, and Z. Dembic. 1992. IFN-γ receptor-Ig fusion proteins: half-life, immunogenicity and in vivo activity. *J. Immunol. 149: 4096.*
40. Paul, M. S., M. Aegerter, K. Cepek, M. D. Miller, and J. H. Weis. 1990. The murine complement receptor gene family. III. The genomic and transcriptional complexity of the *Crry* and *Crry-ps* genes. *J. Immunol. 144:1988.*
41. Linsley, P. S., P. M. Wallace, J. Johnson, M. G. Gibson, J. L. Greene, J. A. Ledbetter, C. Singh, and M. A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation marker. *Science 257:792.*
42. Tang, W. W., L. Feng, J. L. Vannice, and C. B. Wilson. 1994. Interleukin-1 receptor antagonist ameliorates experimental anti-glomerular basement membrane antibody-associated glomerulonephritis. *J. Clin. Invest. 93:273.*
43. Mulligan, M. S., K. J. Johnson, R. F. Todd III, T. B. Issekutz, M. Miyasaka, T. Tamatani, C. W. Smith, D. C. Anderson, and P. A. Ward. 1993. Requirements for leukocyte adhesion molecules in nephrotoxic nephritis. *J. Clin. Invest. 91: 577.*
44. Wu, X., J. Pippin, and J. B. Lefkowith. 1993. Attenuation of immune-mediated glomerulonephritis with an anti-CD11b monoclonal antibody. *Am. J. Physiol. Renal Fluid Electrolyte Physiol. 264:F715.*
45. Wu, X., A. J. Wittwer, L. S. Carr, B. A. Crippes, J. E. DeLarco, and J. B. Lefkowith. 1994. Cytokine-induced neutrophil chemoattractant mediates neutrophil influx in immune complex glomerulonephritis in rat. *J. Clin. Invest. 94:337.*
46. Feng, L., Y. Xia, T. Yoshimura, and C. B. Wilson. 1995. Modulation of neutrophil influx in glomerulonephritis in the rat with anti-macrophage inflammatory protein-2 (MIP) antibody. *J. Clin. Invest. 95:1017.*
47. Wu, X., M. H. Helfrich, M. A. Horton, L. P. Feigen, and J. B. Lefkowith. 1994. Fibrinogen mediates platelet-polymorphonuclear leukocyte cooperation during immune-complex glomerulonephritis in rats. *J. Clin. Invest. 94:928.*
48. O'Meara, Y. M., Y. Natori, A. W. M. Minto, D. J. Goldstein, E. C. Manning, and D. J. Salant. 1992. Nephrotoxic antiserum identifies a β_1 integrin on rat glomerular epithelial cells. *Am. J. Physiol. Renal Fluid Electrolyte Physiol. 262:F1083.*
49. Wilson, C. B. 1996. Renal response to immunologic glomerular injury. In *The Kidney*. B. M. Brenner, ed. W. B. Saunders, Philadelphia, p. 1253.
50. Wang, Y., S. A. Rollins, J. A. Madri, and L. A. Matis. 1995. Anti-C5 monoclonal antibody therapy prevents collagen-induced arthritis and ameliorates established disease. *Proc. Natl. Acad. Sci. USA 92:8955.*