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Inhibition of the CD40-CD40ligand pathway prevents murine membranous glomerulonephritis

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Inhibition of the CD40-CD40ligand pathway prevents murine membranous glomerulonephritis. Several forms of glomerulonephritis are induced by antibodies against self or foreign antigens. Normal B lymphocyte antibody production requires T cell costimulatory signals provided in part by T cell surface expression of gp39/CD40ligand (CD40L) that engages the B cell receptor CD40 and induces B cell differentiation and immunoglobulin class switching. We assessed the effect of disrupting the CD40L-CD40 costimulatory pathway, using a CD40-Ig fusion protein, on the development of membranous glomerulonephritis (MGN) in the mouse. MGN is induced by mouse antibodies that recognize and bind to exogenously administered rabbit anti-mouse renal tubular brush border (RbAMBB) IgG immobilized in the glomerular capillary wall. MGN did not occur in nude mice, showing the need of the T cell function. C57Bl/10 mice immunized with RbAMBB and treated with CD40-Ig fusion protein displayed a delayed autologous response and absence of MGN lesions, while control fusion proteins failed to prevent the development of the disease. These observations provide evidence that disruption of the CD40-CD40L costimulatory pathway can prevent the development of MGN by suppressing T cell-dependent antibody production.

Thymus-dependent humoral immune response requires an articulate dialogue between T and B lymphocytes. T cells provide stimulatory signals for B lymphocyte function and antibody production partly through release of soluble cytokines [1] and partly through cell surface receptors that recognize specific ligands on B cells during cell-cell contact [2, 3]. The critical role of physical T cell-B cell interaction in T cell-dependent antibody production has been clearly demonstrated by the observation that combinations of cytokines alone cannot replace physical contact in inducing B cell proliferation and differentiation [4–7]. A variety of other approaches have subsequently confirmed this view [3, 8, 9]. Cell-cell interaction is required for T cell antigen receptor recognition of foreign peptides presented by B cell MHC class II molecules. However, physical association also allows interaction between several counter-receptors that regulate both T and B cell responses [8, 9]. Some of these counter-receptors, including LFA-1-ICAM-1, primarily facilitate intercellular adhesion, whereas others, including CD4-MHC II, LFA3-CD2 and B7-CD28 function as accessory signaling molecules that facilitate mutual T cell-B cell stimulation by reducing the threshold of lymphocyte response to antigen or by transducing stimulatory signals [9–11]. The receptor-ligand pair composed of the B cell-associated receptor CD40, and its T cell ligand CD40L/gp39, has recently been shown to play a key role in the regulation of T cell-dependent antibody production [12–15].

CD40 is a 47 kDa cell surface glycoprotein expressed on most mature and activated B cells [16], related to the TNF receptor family of cell surface molecules [17]. Recently, a natural ligand of CD40, CD40L/gp39, has been identified and found to be a type II integral membrane protein, related to TNF and ligands of CD30, CD27, 4-1BB and Fas [18]. Cell surface expression of CD40L/gp39 in normal T cells is transient, occurring shortly after activation and persisting for only a few hours [19–21]. *In vitro*, both soluble CD40-immunoglobulin fusion protein (CD40Rg, for receptorglobulin) and mAb to CD40L/gp39 block T cell-dependent B cell proliferation, Ig production and Ig-class switching [12, 20, 22]. Recombinant CD40L/gp39 and mAb to CD40, on the other hand, induce these events in the presence of cytokines [20, 22–24]. Interestingly, B cells cultured with IL-4 and triggered by anti-CD40 mAb produce IgE and IgG4, whereas in the presence of IL-10, they preferentially synthesize IgG, IgA and IgM [25, 26]. The physiologic importance of the CD40-CD40L/gp39 interaction was discovered through the elucidation of the underlying defect in a severe form of human immunodeficiency known as the hyper IgM syndrome (HIM). HIM is characterized by overproduction of IgM but absence of IgG, IgA and IgE, and is accompanied by severe recurrent infections. Recent work has shown that T cells from HIM patients express mutated CD40L/gp39 that cannot interact with CD40 on B cells [27–29]. B lymphocytes from these patients are normal and display appropriate immunoglobulin class switching upon stimulation with recombinant wild type CD40L/gp39 [28, 30].

Inappropriate antibody production against self-antigens as well as normal antibody production against foreign antigens that fail to be eliminated from the organism may lead to a variety of disease states. Human idiopathic membranous glomerulonephritis (MGN) is an antibody-mediated disease of unknown etiology that may lead to renal failure. Morphologically, it is characterized by glomerular subepithelial deposits of immune complexes, severe basement membrane thickening, and little or no inflammatory infiltrate [31]. Treatment is typically based on administration of steroids and other immunosuppressive drugs with controversial results [32].

The possibility to genetically manipulate cell surface receptors

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that mediate cell-cell interactions critical to immune responses offers a potentially powerful means to selectively abrogate undesirable T and/or B cell activity. In the present work, we have explored the possibility to use soluble CD40-Ig fusion protein (CD40Rg) to prevent MGN. Mice injected with purified rabbit-anti-mouse pronase-digested renal tubular brush border IgG (RbAMBB) develop glomerulonephritis that mimicks the morphologic lesions of human MGN, providing a suitable animal model of the disease [33]. We show that early administration of CD40-Ig fusion protein can prevent the development of MGN in the murine model. The ability of CD40Rg to block development of renal lesions provides evidence for CD40-CD40L/gp39 signaling in MGN, and suggests that inhibition of this co-stimulatory pathway may be useful in the treatment of antibody-mediated disease.

Methods

Animals

Eight- to nine-week-old C57Bl/10 and C57Bl/6J nude (nu/nu) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Adult male New Zealand White rabbits were purchased from Charles River Laboratories (Wilmington, MA, USA).

Preparation of RbAMBB

Tubular brush border fraction was extracted from mouse kidney cortices minced and sieved through a 90 μ stainless steel sieve, according to the method of Assmann et al [33]. Sieved material was centrifuged at 400 g for 10 minutes and the cell-pellet discarded. The supernatant was then centrifuged at 78,000 g and the resulting pellet washed three times with distilled water by centrifugation at 78,000 g. The pellet, containing the tubular fraction, was incubated at 37°C for two hours with 30 mg pronase (Calbiochem-Behring Corp., La Jolla, CA, USA) in 60 ml of a 0.8% NaCl-0.02 M Tris HCl buffer, pH 7.8, centrifuged at 100,000 g for one hour and lyophilized. Four rabbits were immunized with 20 mg of pronase-digested tubular brush border fraction in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO, USA) and boosted four weeks later with 10 mg of the same material in incomplete Freund's adjuvant (Sigma). Rabbits were bled two weeks following the boost injection and the IgG fraction from the pooled sera prepared as described [33]. IgG from normal rabbit sera (NRb IgG) were used as a control.

Monoclonal antibodies and immunofluorescence microscopy

Fluorescein (FITC)-conjugated rat anti-mouse CD8a was from Biosource (Camarillo, CA, USA); rat anti-mouse CD4 and rat anti-mouse Thy 1.2 were from Pharmingen (San Diego, CA, USA). Mouse anti-human Fc-specific IgG was from Sigma. Irrelevant IgG_{2b} mAb L14 anti-simian virus 40 large T antigen (gift of Dr. Ed Harlow, Massachusetts General Hospital, Charlestown, MA, USA) was used as control and is referred to as IgG_{2b} mAb. FITC-conjugated goat anti-rabbit IgG, rabbit anti-mouse IgG and rabbit anti-mouse C3 were purchased from Cappel Laboratories (Downington, PA, USA). The goat anti-mouse IgG was absorbed with rabbit IgG, and its specificity was confirmed by absence of binding to kidneys of mice sacrified one day after injection of rabbit anti-rat glomerular basement membrane antiserum [34], while staining with FITC-goat anti-rabbit IgG showed marked linear deposits of rabbit IgG on the glomerular basement membrane.

Table 1. Experimental design

Group	N of mice	Passive immunization (day 0)	Treatment	Days of treatment
I	6	none	none	—
II	6	NRb IgG	none	—
III	10	RbAMBB	PBS	0–40
IV	3	RbAMBB	CD8mRg	0–40
V	6	RbAMBB	IgG_{2b} mAb	0–40
VI	7	RbAMBB	CD40mRg	0–40
VII	6	RbAMBB	CD40mRg	10–40
VIII	6	RbAMBB	none	—

Soluble fusion proteins (CD40mRg and CD8mRg) and the irrelevant IgG_{2b} mAb were injected at the dose of 50 μg in 150 μl of PBS on day 0 and every other day subsequently. Group VIII was formed by C57B1/6J (nu/nu) nude mice. All the animals were sacrificed 40 d after immunization. Abbreviations are: NRb IgG, normal rabbit IgG; RbAMBB, rabbit anti-mouse brush border IgG; CD8mRg, CD8 murine receptorglobulin; CD40mRg, CD40 murine receptorglobulin (additional explanations are in **Methods**).

All animals were nephrectomized at autopsy and the tissue frozen in liquid nitrogen. Cryostat-cut 5 μm tissue sections were mounted onto slides and stained with FITC conjugated antibody, washed several times in PBS, and examined under an epifluorescence microscope. The intensity of staining was graded on a scale from 0 to ++++ (0, absent; +, minimal and focal in amount and extent; ++, moderate and focal; +++, marked and diffuse; ++++, very marked and diffuse). Semiquantitative photometric immunofluorescence measurement of deposits of rabbit IgG present in the glomeruli was performed on 300 randomly-selected individual glomeruli at a magnification of 600× with a Nikon photometer Model UF x 11A. Fluorescence intensity was expressed as the reciprocal of the exposure time.

Electron microscopy

Small fragments of renal cortex (3 mm^3) were fixed in Karnovsky's paraformaldehyde-glutaraldehyde solution [35], post-fixed in 1% osmium tetroxide and embedded in Epon 812. Thin sections stained with uranyl acetate and lead citrate were studied with a Hitachi electron microscope.

Flow cytometry

FACS analysis was performed on Ficoll-separated splenocytes from all animals included in Groups II-VII for Thy1.2, CD4 and CD8 cell surface expression. In addition, the murine Th2 cell line D10.G4.1 (ATCC, Rockville, MD, USA), unstimulated or stimulated with 10 μg/ml Concanavalin-A (Sigma) for six hours, and unstimulated and 10 μg/ml Concanavalin A-stimulated, Ficoll-separated, splenocytes from normal C57Bl/10 mice, were incubated with CD40Rg for 45 minutes at 4°C in PBS, followed by a secondary FITC-conjugated goat-anti-mouse or goat anti-human affinity purified antibody, washed and subjected to FACS analysis (Becton-Dickinson, Mountainview, CA, USA). To avoid non-specific Fc receptor-dependent binding, splenocytes were preincubated for one hour at 4°C with 100 μg/ml purified mouse IgG (Cappel Laboratories) in PBS, washed and then stained.

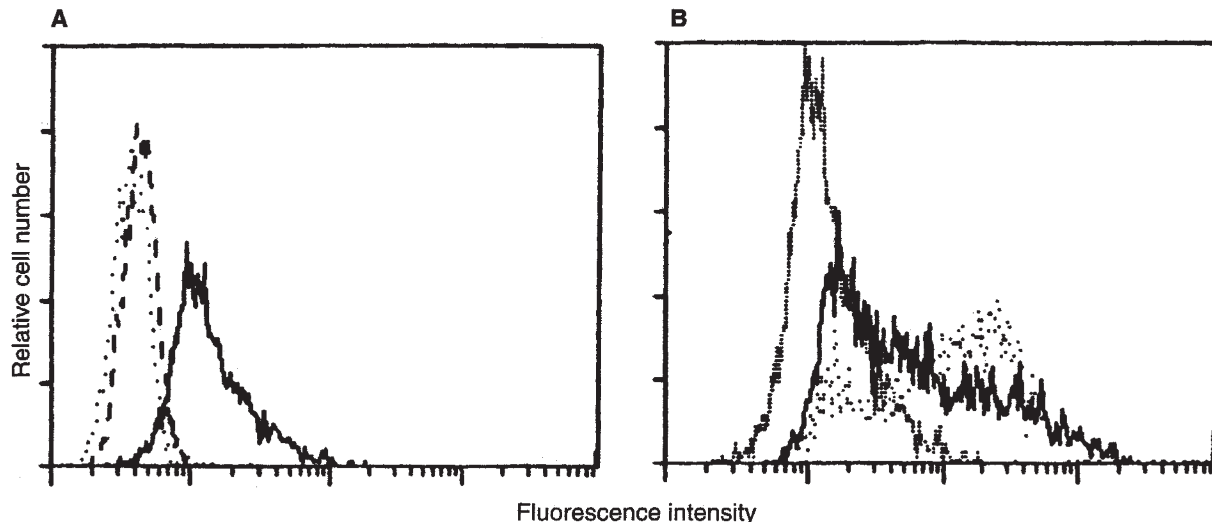

Fig. 1. *Binding of CD40Rg to murine lymphocytes.* (**A**) Murine D10.G4.1 T cells stimulated with 10 μg/ml Concanavalin A for six hours, were incubated with PBS (dotted line), 10 μg/ml CD40mRg (solid line) or 10 μg/ml CD8mRg (dashed line), followed by an FITC-labeled goat anti-mouse IgG. (**B**) Unstimulated murine splenocytes were incubated with 10 μg/ml human IgG (broken line) or with 10 μg/ml CD40hRg (solid line), and splenocytes stimulated with 10 μg/ml Concanavalin A for six hours were incubated with CD40hRg (dotted line) followed by an FITC-labeled goat anti-human IgG that does not cross-react with mouse IgG.

Detection of mouse antibodies to rabbit IgG

The autologous antibody response phase of all animals in the experimental groups was assessed by determination of serum mouse anti-Rb IgG levels at days 0, 7, 21 and 40 after immunization in an ELISA. Ninety-six-well microtiter plates were coated with 1 μg/ml of purified rabbit IgG in PBS/0.02% sodium azide overnight at 4°C. The plates were washed with PBS-0.05% Tween-20 and incubated with PBS-2% skimmed milk for 30 minutes at room temperature (RT). After two washes with PBS-0.05% Tween-20, serial 1:50 to 1:500 dilutions of serum samples (100 μl/well) were added. Following an overnight incubation at 4°C, the plates were washed and exposed for four hours to alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma), diluted 1:4000. After three washes with PBS-0.05% Tween-20, p-nitrophenyl phosphate (Sigma) dissolved in a 0.1 M glycine/10 mM $MgCl_2$ solution, pH 9.4, was added. Following three hours of incubation at RT, the colorimetric reaction was read at 405 nm in an EL-310 Microplate Autoreader (Biotek Instruments Inc., Winooski, VT, USA).

Proteinuria and PBL counts

Proteinuria at day 40 after immunization was measured using a Protein Assay Kit (Sigma) based on Peterson's modification of the micro-Lowry method with a minimal sensitivity of 50 μg/ml. Samples of peripheral blood were collected in heparinized tubes at the end of the treatment (day 40). Peripheral blood leukocytes (PBL) were counted using a hemocytometer (Fisher Scientific Co., Pittsburgh, PA, USA).

Development and production of soluble recombinant fusion proteins

Soluble receptor globulins were developed by genetic fusion of sequences encoding the extracellular region of murine CD40 to genomic DNA sequences containing exons encoding the hinge, CH2 and CH3 domains of murine IgG_{2b} or human IgG_1 (CD40mRg and CD40hRg, respectively) [36]. Synthetic oligonucleotide primers complementary to the 5′ and 3′ extremities of the nucleotide sequence encoding the extracellular domain of murine CD40 were used to PCR-amplify mouse CD40 from cDNA derived from the WEHI-231 murine B cell line (American Type Culture Collection, Rockville, MD, USA) stimulated for four hours with 5 μg/ml pokeweed mitogen (Sigma). The forward and reverse primers were designed to contain an *Xho*I and a *Bam*HI site, respectively, to facilitate in-frame ligation to Ig expression vectors. Nucleotide sequences of the primers were:

Forward: 5′ CAC GGG CTC GAG ATG GTG TCT TTG CCT CGG CTG TGC GCG CTA TGG 3′

Reverse: 5′ CGC GGG ATC CCG GGA CTT TAA ACC ACA GAT GAC 3′

Thirty amplification cycles at 94°C/1 minute/60°C/2 minutes/72°C/3 minutes were performed using amplitaq polymerase (Perkin-Elmer) and buffers recommended by the vendor. Amplified cDNA was subjected to *Xho*I/*Bam*HI digestion and ligated to *Xho*I/*Bam*HI-cut human IgG_1 expression vector. For insertion into the murine IgG_{2b} expression vector, the CD40hRg construct was digested with *Mlu*I and *Bam*HI and the insert ligated to *Mlu*I/*Bam*HI-cut murine IgG_{2b} expression vector.

Plasmids containg sequences encoding CD40 receptorglobulins bearing murine and human IgG Fc were introduced into COS cells by electroporation at 250 V/960 μF using a Biorad Gene Pulser (Richmond, CA, USA). Serum-free supernatants were collected five to seven days post-transfection and soluble fusion proteins purified on protein A sepharose as previously described [36]. A soluble CD8mRg fusion protein encoded by sequences specific for the extracellular domain of human CD8, previously shown to be non-reactive with murine tissues [37], and murine IgG_{2b} Fc was prepared using the same approach and served as a control. Purified soluble fusion proteins were analyzed by SDS/10% PAGE under reducing conditions. Gels were stained with Coomassie blue.

Table 2. Immunofluorescence findings

		Groups							
		I	II	III	IV	V	VI	VII	VIII
Rb IgG[a]	GPCW	0	0	++++	++++	++++	0	++/+++	0
	M	0	0	0	0	0	0	0	0
	BC	0	0	++	++	++	++	+/++	++
	T	0	0	+/++	+	+	+	+	+
M IgG	GPCW	0	0	++++	++++	++++	0	++/+++	0
	M	++	++	++/+++	++	++	++/+++	++/+++	+++
	BC	0	0	+/0	+/0	0	+/0	+/0	+/0
	T	0	0	+/0	+/0	+/0	0	+/0	0
M C3	GPCW	0	0	+	+/++	+/++	0	+/0	0
	M	++	++	++	++	++	++	++/+++	++
	BC	+++	+++	+++	+++	+++	+++	+++	+++
	T	+++	+++	+++	+++	+++	+++	+++	+++

Abbreviations are: Rb IgG, rabbit IgG; M IgG, mouse IgG; M C3, mouse C3; GPCW, glomerular peripheral capillary walls; M, mesangium; BC, Bowman's capsule; T, tubular basement membrane.

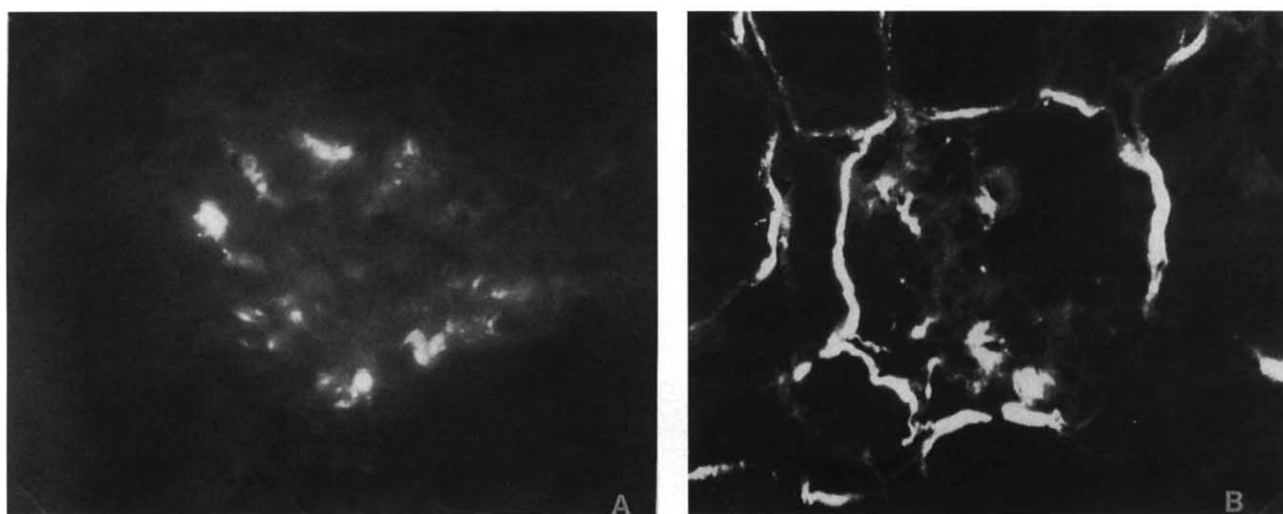

Fig. 2. *Glomerular immunofluorescence findings in a naive C57Bl/10 mouse.* (**A**) Coarse deposits of mouse IgG in the mesangium. (**B**) Focal deposits of mouse C3 in the mesangium, Bowman's capsule and tubular basement membranes. ×400.

Induction of MGN and experimental design

C57Bl/10 and C57Bl/6J nude (nu/nu) mice were surgically mononephrectomized under sterile conditions and allowed to fully recover for at least three weeks. On day 0 of the experiment, all animals were immunized with a single injection of 7.5 mg RbAMBB or NRb IgG in 300 μl of PBS into the tail vein. Experimental groups are summarized in Table 1. Mice in Groups III to VI were injected every other day, from day 0 to day 40, with 50 μg CD40mRg, CD8mRg or IgG_{2b} mAb in 150 μl PBS or with 150 μl PBS alone. Mice in Group VII were injected from day 10 to day 40 with 50 μg CD40mRg in 150 μl PBS every other day. All of the mice in groups III to VII received the first ten injections i.v. (tail vein), and the subsequent injection i.p. All animals were sacrificed at day 40. In order to establish whether injections of CD40mRg modified the binding of RbAMBB to the kidneys, additional mice, injected like mice in Groups II and VI, were sacrified at day 1, and the immunofluorescence patterns were compared.

Statistics

Statistical analysis, when applicable, was performed using Statview IV software (Abacus Concepts, Berkeley, CA, USA) on a Macintosh SE computer (Apple Computer, Inc., Cupertino, CA, USA). Differences between groups were compared by one way analysis of variance and unpaired *t*-test.

Results

Characterization of soluble recombinant murine CD40Rg

Murine CD40 extracellular domain-specific sequences were amplified by PCR from WEHI 231 B cell cDNA and ligated to genomic sequences containing murine IgG_{2b} Fc or human IgG_1 Fc exons [36]. Fusion proteins were recovered from COS cells transfected with each of the constructs in a transient expression system, and purified on protein A beads as described previously [36]. Development of CD8mRg was performed by translocating

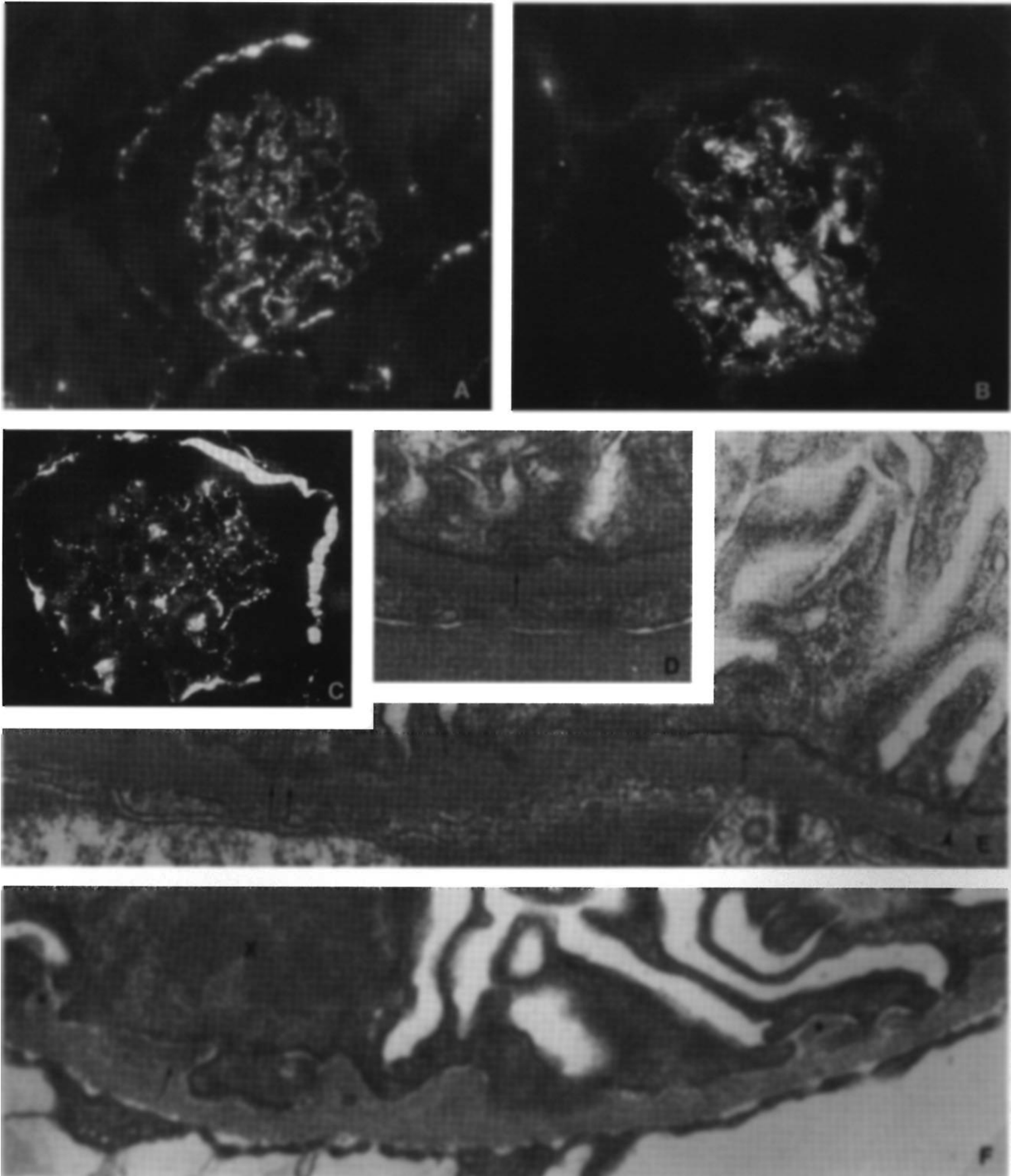

Fig. 3. *Immunofluorescence and electron microscopy of glomeruli from a C57Bl/10 mouse injected with RbAMBB and treated for 40 days with CD8mRg (Group IV).* (**A**) Granular deposits of rabbit IgG in the peripheral glomerular capillary walls, in Bowman's capsule and in tubular basement membranes. (**B**) Diffuse, granular deposits of mouse IgG in the peripheral glomerular capillary walls; coarse deposits are also present in the mesangium. (**C**) Granular deposits of mouse C3 in the peripheral glomerular capillary walls; coarse deposits are also present in the mesangium and in Bowman's capsule. (**D** and **E**) Electron micrographs showing deposits of foreign material between the basement membrane and the foot processes (arrow) or in the filtration slits (arrowhead). (**F**) Electron micrograph showing lesions of the glomerular basement membrane similar to "spikes" of type 2 to 3 human MGN (small asterisks), deposits of foreign material (arrow) and fusion of epithelial foot processes. A–C, ×400; D–F, ×40,000.

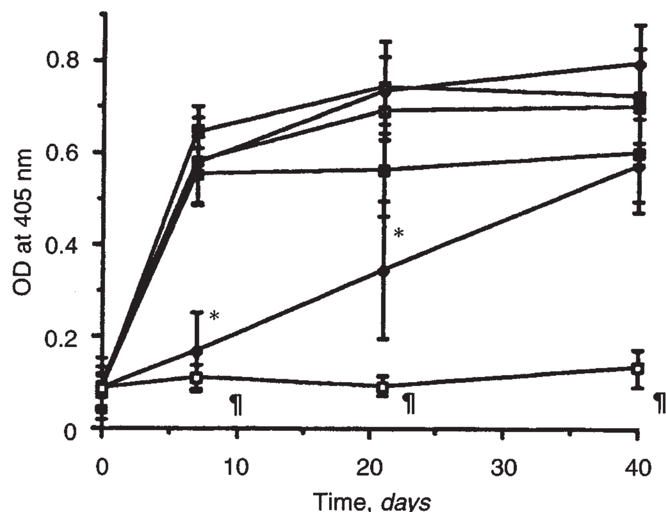

Fig. 4. *Circulating antibodies to Rb IgG detected by ELISA.* At day 7 and 21, the titers of antibodies of Group VI mice were significantly lower than those in Group III and V (*$P < 0.05$). The difference between the titers in Group VIII and those in Groups III and V was always statistically significant (¶$P < 0.05$). Naive mice had an average titer of 0.082 ± 0.020, which was considered as background in the assay. Symbols are: (—⊡—) Group III, (—◆—) Group IV, (—■—) Group V, (—◆—) Group VI, (—■—) Group VII, (—□—) Group VIII.

the extracellular domain of human CD8 from an expression vector containing human IgG_1 Fc sequences [36] to the corresponding plasmid containing murine IgG_{2b} sequences. SDS/PAGE analysis under reducing conditions showed that purified CD40mRg, CD40hRg and CD8mRg migrated as single bands of 50, 45 and 55 kD, respectively (data not shown).

To determine ligand recognition by CD40mRg, the murine helper T cell line D10.G4.1 was subjected to a six hours stimulation with 10 μg/ml Concanavalin A, and tested for CD40mRg binding by flow cytometry. CD40mRg specifically bound to stimulated but not to unstimulated D10.G4.1 cells (Fig. 1A). Similarly, CD40hRg bound murine Concanavalin A-stimulated splenocytes but reacted weakly with unstimulated splenocytes (Fig. 1B).

Development of MGN

Murine MGN was induced according to the method of Assmann et al [33] by administering a single dose of 7.5 mg of RbAMBB intravenously. To establish the chronology of the lesions, 12 C57Bl/10 mice injected with RbAMBB were subdivided into four groups of three mice each that were sacrificed on days 1, 10, 30 and 40 following injection. Kidney sections from animals in each group were prepared and examined by immunofluorescence. Specimens obtained at days 30 to 40 were also studies by electron microscopy. On day 1, linear deposits of rabbit IgG were seen in the peripheral glomerular capillary walls, in the endothelium of peritubular capillaries and larger vessels and along the brush border of proximal tubules (data not shown). Linear, segmental deposits of mouse C3 were present in the tubular basement membrane and in Bowman's capsule (see Groups I and II). Deposits of mouse IgG were present in the mesangium (see Groups I and II), but were not detectable in the peripheral capillary walls. On day 10, the deposits of rabbit IgG in the peripheral glomerular capillary walls were still linear, and were associated with faint deposits of mouse IgG (data not shown). C3 was present principally in the mesangium. The tubular brush border was no longer stained. On days 30 and 40, diffuse granular deposits of rabbit IgG, mouse IgG and mouse C3 were visible in the peripheral glomerular capillary walls, corresponding to dense deposits in the subepithelial part of the glomerular basement membrane and "spikes" observed by electron microscopy (see Groups III, IV and V). The tubular brush border appeared normal.

Groups I and II

These groups were comprised of normal mice (Group I) and mice injected with normal rabbit IgG (Group II). The experimental design is shown in Table 1. The results obtained by immunofluorescence technique at day 40 are summarized in Table 2. In Groups I and II, that provide negative controls for disease development, coarse deposits of mouse IgG were present in the mesangium (Fig. 2A). Linear segmental deposits of mouse C3 were localized in tubular basement membranes and in Bowman's capsules (Fig. 2B). Some dense deposits were found by electron microscopy in the mesangial matrices. These deposits were present in almost all glomeruli and tubules. In contrast, the peripheral glomerular capillary walls, the interstitium and both small and large vessels were consistently normal. Renal deposits of rabbit IgG were absent. Mouse anti-rabbit IgG were never detectable in sera of Group I animals. Forty days after immunization, the average titer of mouse anti-rabbit IgG in the sera of Group II mice was 0.72 ± 0.14 OD. Proteinuria was 32 ± 4.2 mg/dl and 30 ± 8 mg/dl in Group I and II animals, respectively. PBL count of Group II mice was 7.2 ± 0.6 × $10^3/mm^3$ and splenocyte FACS analysis showed 28.5 ± 3.4% Thy 1.2^+, 18.4 ± 2.2% $CD4^+$, 7.5 ± 0.2% $CD8^+$ cells.

Groups III, IV and V

Mice were injected with RbAMBB and treated with PBS (Group III), CD8mRg (Group IV), or the irrelevant mouse monoclonal antibody IgG_{2b} (Group V). Some mice were injected with RbAMBB and PBS, and sacrificed 1, 2 and three days thereafter. On day 1 marked deposits of rabbit IgG were found in glomerular capillary walls, in the brush border and tubular basement membranes. Deposits of mouse C3 were absent. On days 2 and 3 the deposition of rabbit IgG progressively decreased in the glomerular capillary walls and in the brush border, while granular deposits appeared in tubular basement membranes (not shown). At day 40 mice of Groups III, IV and V developed diffuse granular deposits of rabbit and mouse IgG in glomerular peripheral capillary walls, while murine C3 was detectable in only 30% of the animals (Fig. 3A–C). Granular deposits of rabbit IgG, with small amounts of mouse IgG, were present along the basement membranes of proximal tubules and in Bowman's capsules. Electron microscopy revealed small dense deposits in the subepithelial part of the glomerular basement membranes and "spikes" similar to those seen in type 2 to 3 human MGN [31] (Fig. 3D–F). Mesangial deposits were comparable to those observed in Group I and II animals. Antibodies to rabbit IgG became detectable in the serum of these mice at day 7 and reached a peak at day 21 (Fig. 4). Proteinuria was 33.5 ± 9.4 mg/dl (Group III), 29.6 ± 7.3 mg/dl (Group IV) and 27 ± 4.6 mg/dl (Group V) and was comparable to those in normal mice (Group I) and in mice injected with normal RbIgG (Group II). This observation is

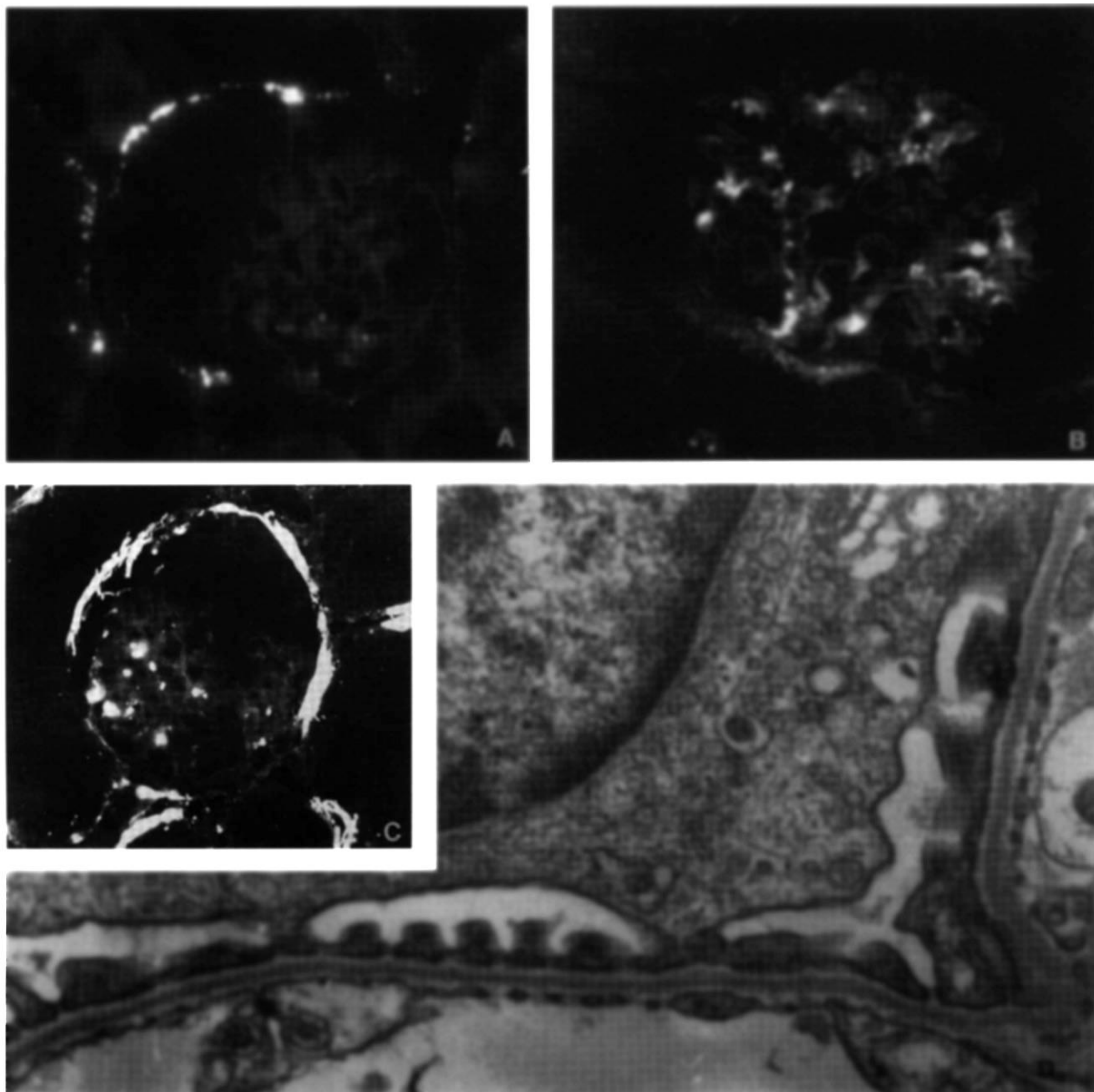

Fig. 5. *Immunofluorescence and electron microscopy in a C57Bl/10 mouse injected with RbAMBB and given CD40mRg from day 0 to day 40.* (**A**) Granular deposits of rabbit IgG in the Bowman's capsule but not in glomerular peripheral capillary walls. (**B**) Deposits of mouse IgG in the mesangium, but not in glomerular peripheral capillary walls. (**C**) Deposits of mouse C3 in the mesangium, in Bowman's capsule and in tubular basement membranes, but not in glomerular peripheral capillary walls. (**D**) Electron micrograph showing normal glomerular capillary walls. A–C, ×400; D, ×40,000.

consistent with that of Assmann et al [33]. The PBL count was 7.5 $\pm$ 1.2 $\times$ $10^3/mm^3$, 6.8 $\pm$ 1.1 $\times$ $10^3/mm^3$ and 7.0 $\pm$ 0.9 $\times$ $10^3/mm^3$ in Group III, IV and V, respectively. FACS analysis showed the following splenocyte phenotype: Group III, 29.2 $\pm$ 4.4% Thy 1.2^+, 18.4 $\pm$ 4.2% $CD4^+$, 8.3 $\pm$ 1.0% $CD8^+$ cells; Group IV, 30.2 $\pm$ 2.4% Thy 1.2^+, 22.6 $\pm$ 3.8% $CD4^+$, 8.0 $\pm$ 0.7% $CD8^+$ cells; Group V, 26.8 $\pm$ 3.1% Thy 1.2^+, 20.9 $\pm$ 4.0% $CD4^+$, 6.9 $\pm$ 1.2% $CD8^+$ cells.

Group VI

Mice were immunized with RbAMBB and treated with CD40mRg from day 0 to day 40. In mice sacrificed one day after injection of RbAMBB and CD40mRg the deposition of rabbit IgG in glomerular capillary walls and tubular brush border was unchanged, as compared to mice injected with RbAMBB and PBS which were sacrified at the same interval of time (not shown). At day 40 tissue examination by immunofluorescence and electron microscopy revealed normal glomerular peripheral capillary walls in all animals (Fig. 5A–D). The virtual absence of rabbit IgG, compared to mice in Groups III to V, was confirmed by semi-quantitative photometric analysis (Fig. 6). Granular deposits of rabbit IgG were found in the basement membranes of proximal tubules and in Bowman's capsules. Staining for mouse IgG and C3

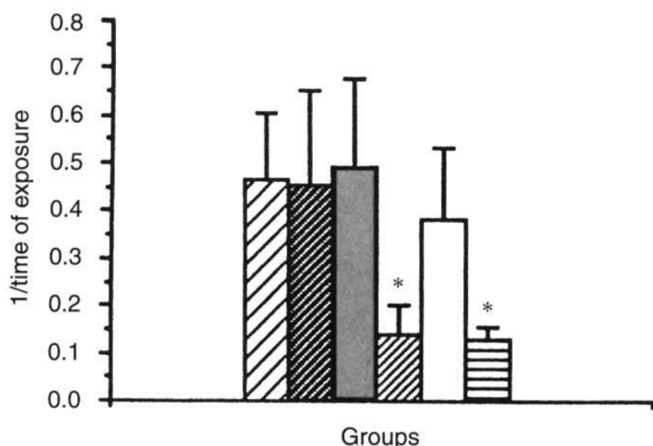

Fig. 6. *Glomerular photometric analysis of the intensity of fluorescence staining for Rb IgG at day 40, expressed as the reciprocal of the exposure time(s).* Values in Groups VI and VIII were significantly lower than those in control Groups III, IV and V ($*P < 0.05$). Symbols are: (▨) Group III, (▩) Group IV, (▤) Group V, (▨) Group VI, (□) Group VII, (⊟) Group VIII.

in the mesangium did not differ appreciably from that seen in mice within Groups I to V. During the first three weeks following immunization, mouse anti-rabbit IgG antibody levels were significantly lower than in Group III, IV and V animals (Fig. 4), but rose to comparable titers thereafter. Proteinuria was 27.7 ± 5.3 mg/dl PBL count was $7.1 \pm 0.9 \times 10^3/mm^3$. Splenocyte phenotype was comparable to that of Group III, IV and V animals, with 27.5 ± 3.4% Thy 1.2^+, 18.4 ± 3.6% $CD4^+$, and 7.5 ± 2.2% $CD8^+$ cells.

Group VII

To determine the effect of CD40mRg when administered following disease onset, this group of animals received CD40mRg, their injections beginning on day 10 after RbAMBB immunization when anti-RbIgG antibodies were detectable in serum. Two mice within this group had significantly fewer immune deposits in the peripheral glomerular capillary walls (Fig. 7A and B), whereas glomerular deposits in the other four were comparable to those in Group III, IV and V animals. Although the levels of circulating mouse antibodies to rabbit IgG were lower than in Groups III, IV and V, the difference was marginal (Fig. 4). Proteinuria (29.7 ± 8.4 mg/dl), PBL counts and FACS analysis of splenocyte populations were comparable to those of normal mice.

Group VIII

Nude mice injected with RbAMBB comprised Group VIII. The observations that RbAMBB-induced MGN appears to depend on mouse anti-rabbit IgG production, and that it can be prevented by interfering with T cell-dependent B cell stimulation along the CD40-CD40L/gp39 axis, predict that production of this form of MGN should not be possible in thymus-deficient animals. Accordingly, we administered RbAMBB to a group of nude mice and assessed any resulting glomerular lesions. Deposits of rabbit and mouse IgG and C3 in the glomerular peripheral capillary walls were minimal or absent (Fig. 7 C, D). By electron microscopy a few gross irregularities were seen in the epithelial profile of the glomerular basement membranes, but diffuse subepithelial deposits of foreign material and "spikes" comparable to those seen in mice of Groups III, IV and V, were absent. Mesangial deposits of mouse IgG and C3 did not differ from those seen in naive C57B1/10 and control mice (Groups III, IV and V). Proteinuria was 28.5 ± 8.5 mg/dl. Circulating antibodies to rabbit IgG were not detectable (Fig. 4).

Discussion

Several recent studies have shown that monoclonal antibodies to, and soluble recombinant forms of, specific lymphoid cell surface receptors that mediate cell-cell interaction and participate in the regulation of lymphocyte response to antigenic stimulus, can be used to manipulate the immune response and to control inflammation. Thus, simultaneous administration of anti-ICAM-1 and anti-LFA-1 mAb has been observed to block graft rejection [38] and rapidly progressive glomerulonephritis [39]. Soluble CTLA-4 prolongs allograft [40] and xenograft [41] survival and inhibits rapidly progressive glomerulonephritis [42]. Soluble L- and P-selectin can respectively block peritoneal neutrophil efflux [43] and prevent acute pulmonary inflammation [44]. An anti-CD40L mAb has been effective in preventing collagen-induced murine arthritis [45]. In the present work we have shown that soluble CD40Rg can prevent the development of MGN in the mouse.

CD40Rg binds CD40L/gp39 on the surface of activated T cells, and may thereby prevent CD40L/gp39-CD40 association during cognate T cell-B cell interaction [12]. Since engagement of CD40 by CD40L/gp39 is required for B cell production of antigen-specific IgG, it seems reasonable to suggest that inhibition of CD40L/gp39-CD40 interaction by CD40mRg resulted in the delay of mouse anti-Rb IgG antibody production in the present model. The observed prevention of MGN by CD40mRg was not due to the Fc portion of the fusion protein, since CD8mRg and an irrelevant isotype-matched murine mAb had no effect on antibody deposition in the glomerular capillary walls. Whether binding of CD40mRg to activated T cells *in vivo* results in their opsonization and lysis or only in prevention of CD40L/gp39-CD40 interaction has not been determined. However, the present results are consistent with the notion that CD40mRg induces selective functional inhibition of T cell-B cell interaction without causing leukopenia or even detectable T cell depletion.

MGN may be induced by antibodies binding to constitutive antigens of glomerular visceral epithelial cells or to exogenous antigens immobilized in the subepithelial region of the glomerular basement membrane [46]. RbAMBB recognizes antigens expressed on the surface of murine glomerular endothelial and visceral epithelial cells, including dipeptidyl peptidase IV [47] and aminopeptidase A [48]. The resulting membrane antigen/rabbit IgG complexes are shed between the epithelial cells and the basement membrane [49]. In mice of Groups III, IV and V progressive glomerular disease is induced by a strong and sustained antibody response to rabbit IgG, which probably cross-links immobilized immune complexes to the glomerular basement membrane [50], resulting in progressive enlargement of the complexes that appear as granular deposits in immunofluorescence and electron microscopy. Studies performed with cationic antigens that become immobilized in glomeruli have shown that cross-linking by antibody is a prerequisite for the persistence of these antigens in the subepithelial region of the glomerular basement membrane [51, 52]. We found that the early binding of rabbit IgG to mouse kidneys was not modified by administration

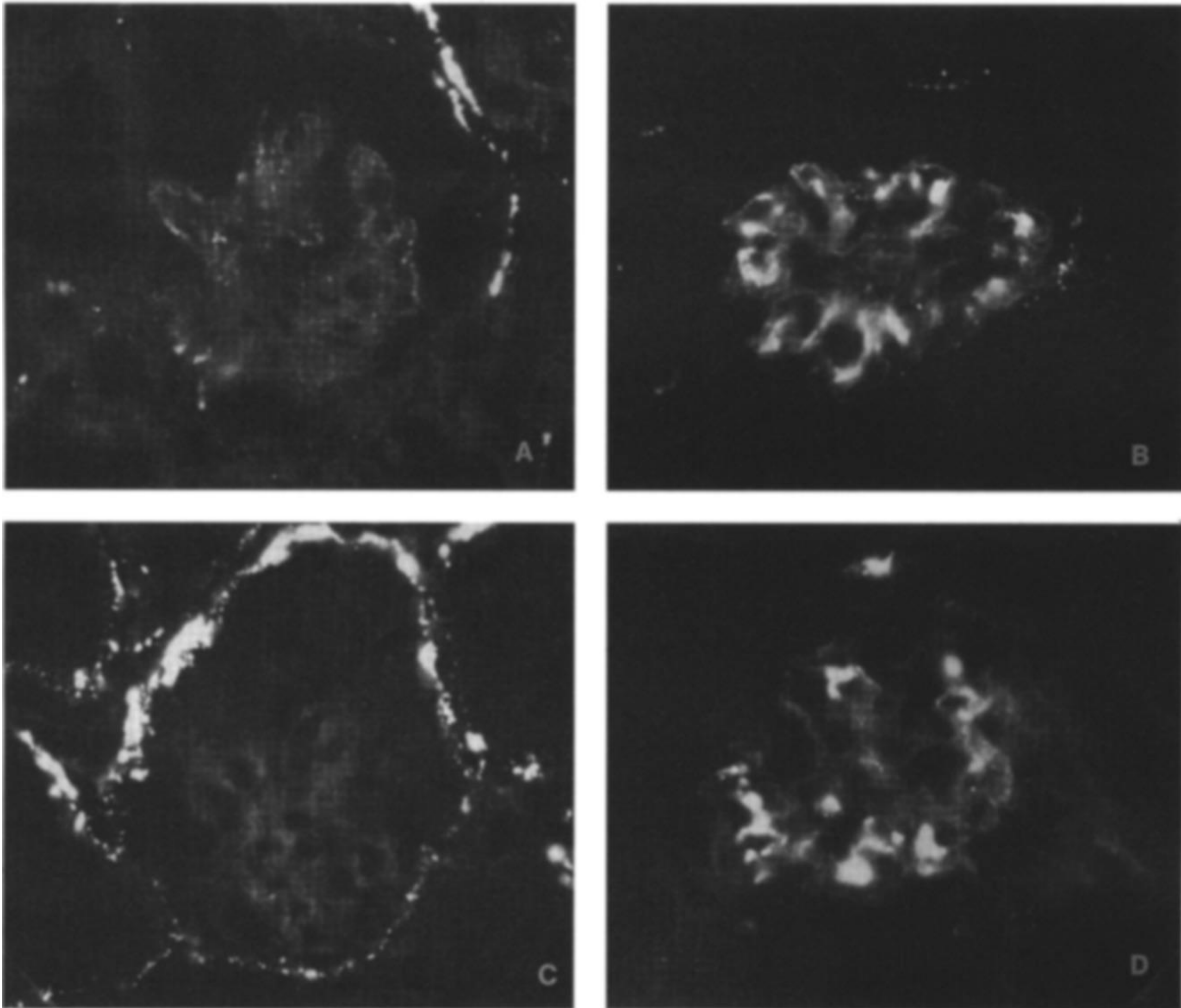

Fig. 7. *Immunofluorescence findings in a C57Bl/10 mouse injected with RbAMBB and given CD40mRg from day 10 to day 40* (**A** *and* **B**)*, and in a nude mouse injected with RbAMBB* (**C** *and* **D**). (A) Moderate granular deposits of rabbit IgG in glomerular peripheral capillary walls with marked deposits in Bowman's capsule. (B) Minimal to moderate granular deposits of mouse IgG in glomerular peripheral capillary walls and in Bowman's capsule, with coarse deposits in the mesangium. (C) Granular deposits of rabbit IgG in Bowman's capsule and in tubular basement membranes, but not in glomerular peripheral capillary walls. (D) Deposits of mouse IgG in the mesangium, but not in glomerular peripheral capillary walls. ×400.

of CD40mRg. Thus our results indicate that the inhibition of the autologous immune response was responsible for the clearance of immune complexes by glomerular visceral epithelial cells [53], and immune deposits did not develop in the peripheral glomerular capillary walls. This interpretation is supported by the observation that MGN did not occur in nude mice, which lack T cells and cannot mount a T cell-dependent antibody response to rabbit IgG [54].

In contrast, despite suppression of the autologous phase, granular deposits of rabbit IgG were found in the tubular basement membranes and in the Bowman's capsules of mice treated with CD40mRg, as well as in control mice (Groups III, IV and V) and in nude mice (Group VIII). We propose that formation of these deposits does not require cross-linking by mouse IgG, and that they are formed by rabbit IgG and plasma membrane antigens of brush border and basolateral membranes. This hypothesis is in agreement with our previous observations of immune deposits in the basolateral compartments of rabbits [55] and rats [56] injected intravenously with anti-brush border antibodies. The immune deposits in Bowman's capsules are probably formed by complexes of rabbit IgG and plasma membrane antigens shed by glomeruli and reabsorbed by the tubules. Regardless of the nature of the immune deposits, their rapid removal from the peripheral glomerular capillary walls in mice treated with CD40mRg contrasts with their persistance in tubular basement membranes and Bowman's capsules, and suggests different mechanisms of clearance in various parts of the nephron.

While CD40mRg has been observed to be a potent inhibitor of MGN development in the murine model, its effectiveness in reversing established disease was less obvious. Two out of six animals did show a marked reduction in rabbit and murine IgG deposits but only a minimal reduction was seen in four others.

This is most likely a reflection of transient CD40L/gp39 expression on activated T cells, suggesting that timing of CD40mRg administration is of paramount importance. If cognate T cell-B cell interaction, and more specifically, mutual CD40L/gp39-CD40 triggering, has already occurred, CD40Rg may no longer be able to prevent high affinity antibody production. Thus, effectiveness, at least in the present model, appears maximal when CD40mRg and antigen are administered simultaneously. Interestingly, the autologous response phase is inhibited transiently by CD40mRg treatment, and anti-Rb IgG levels in treated animals at 40 days are similar to those of untreated controls. Additional support to this view is provided by a recent study on the effect of a soluble CD40 on antibody response *in vivo* [57]. Possible explanations for the transient inhibitory effect include potential alternative routes for regulation of antibody production that might bypass the CD40-CD40L/gp39 axis, and production of antibodies against an immunogenic CD40mRg epitope, that over time inactivate the fusion protein. Importantly, the presence of mouse anti-Rb IgG antibodies, even in high titers at day 40, can no longer induce MGN, since rabbit IgG has been removed by local cellular mechanisms.

The present study provides direct evidence that timely administration of CD40mRg can efficiently inhibit antibody-mediated glomerular disease. Maximal effectiveness of CD40mRg appears to occur during a narrow window of early immune response, corresponding to cell surface expression of CD40L/gp39 [13, 14, 58]. CD40mRg may therefore provide a valuable reagent in studying the pathogenesis of immune-mediated renal disease. Because of the importance of the time of administration with respect to the disease process, as illustrated by the present model, the effectiveness of CD40Rg in clinical situations may depend in part on diagnostic approaches that are able to uncover early phases of disease activity and exacerbation.

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