

Interleukin-6 Receptor Blockage Ameliorates Murine Lupus Nephritis¹

Bryce A. Kiberd²

B.A. Kiberd, Department of Medicine, Queen's University, Kingston, Ontario, Canada

(J. Am. Soc. Nephrol. 1993; 4:58-61)

ABSTRACT

The therapeutic effects of a neutralizing monoclonal antibody (mAb) to the interleukin-6 receptor (IL-6R) were examined in the MRL-lpr/lpr murine lupus nephritis model. Animals (15 wk old) were treated with ip mAb IL-6R for 5 wk. GFR in these mice at the end of this treatment period were comparable to those of congenic strain disease-resistant MRL-+/+ controls treated with rat immunoglobulin G (IgG) (254 ± 61 versus 285 ± 26 $\mu\text{L}/\text{min}$; $P = \text{not significant}$). GFR was significantly ($P < 0.05$) lower in lpr/lpr mice receiving ip rat IgG (disease controls) at the same time (165 ± 76 $\mu\text{L}/\text{min}$). The fractional mesangial volume (Mv) and surface density of open glomerular capillaries (Sv) in mAb IL-6R-treated lpr/lpr and IgG-treated +/+ mice (Mv, 0.21 ± 0.04 and 0.19 ± 0.04 $\mu\text{m}^3/\mu\text{m}^3$; Sv, 0.18 ± 0.01 and 0.20 ± 0.01 $\mu\text{m}/\mu\text{m}^2$, respectively) were similar. However, Mv (0.40 ± 0.04) was significantly higher ($P < 0.001$) and Sv (0.13 ± 0.04) was lower ($P < 0.01$) in IgG-treated lpr/lpr animals. Treatment with mAb IL-6R significantly reduced anti-dsDNA antibody levels after Week 2 of treatment, but these values rebounded at Week 4. The late development of neutralizing antibodies and the increased secretion of IL-6 at Week 4 were likely responsible. Despite these events, neutralizing mAb to the IL-6R proved to be effective therapeutically, as demonstrated by preserved glomerular function and structure.

Key Words: Glomerular morphometry, GFR, cytokines, monoclonal antibody treatment

Systemic lupus erythematosus (SLE) is characterized by polyclonal B-cell activation, autoantibody production, and immune complex deposition (1). The

latter is, at least in part, responsible for inducing a proliferative glomerulonephritis, which can be a significant cause of patient morbidity (2). Interleukin-6 (IL-6) is a pleiotropic cytokine that is elevated in some patients with SLE (3,4). Blocking IL-6 activity *in vitro* with neutralizing antibodies markedly reduces the excessive immunoglobulin and anti-dsDNA antibody production in B cells of patients with active lupus (4,5). IL-6 is also an autocrine growth factor for mesangial cells (6). Increased urine excretion of IL-6 is seen in patients with idiopathic mesangial proliferative glomerulonephritis (7). Transgenic mice overexpressing the IL-6 gene develop a mesangial proliferative glomerulonephritis (8). Therefore, IL-6 may play a role in lupus nephritis because of both enhanced autoantibody production (immune complex deposition) and a stimulus to inappropriate mesangial cell growth.

The purpose of this study was to examine whether blocking IL-6 would be useful therapeutically. IL-6 is elevated in this MRL-lpr/lpr murine SLE model, and these animals develop a progressive proliferative glomerulonephritis with premature death due to this complication (9,10). Specific antagonism of IL-6 was carried out with a neutralizing antibody to the IL-6 receptor (IL-6R). Antibodies to the IL-6R have recently been found to inhibit IL-6-sensitive tumor growth in a mouse plasmacytoma model (11).

METHODS

Female MRL-lpr/lpr and MRL-+/+ mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 6 wk of age. They were housed in our Animal Care Facility and had free access to food and standard chow. At age 15 wk, MRL-lpr/lpr animals were randomly assigned to two groups. Group REC ($N = 6$) received a rat monoclonal antibody (mAb) to the mouse IL-6R (mAb IL-6R; Genzyme, Cambridge, MA) at a dose of 40 to 45 μg ip on days 0, 1, 2, 3, 7, 9, 12, 15, 17, 19, 22, 25, 29, 32, and 36. This schedule was chosen to achieve a systemic antibody level of 2 to 5 $\mu\text{g}/\text{mL}$, which is reported to inhibit cell proliferation by >80% of an IL-6-sensitive cell line supplemented with 1,500 pg/mL of IL-6 (12). Group immunoglobulin G (IgG) ($N = 6$) received rat IgG (Organon Teknika Inc., Scarborough, Ontario, Canada) at a dose and schedule identical to that for REC mice. MRL-+/+ animals served as normal controls (CON; $N = 6$) and received rat IgG, as did IgG MRL-lpr/lpr mice.

¹ Received December 17, 1992. Accepted March 2, 1993.

² Correspondence to Dr. B.A. Kiberd, Division of Nephrology, Rm 5077-Dickson Centre, Victoria General Hospital, 1278 Tower Road, Halifax, Nova Scotia B3H 2Y9, Canada.

1046-6673/0401-0058\$03.00/0

Journal of the American Society of Nephrology

Copyright © 1993 by the American Society of Nephrology

On Day 35, animals were anesthetized with halothane and received [^{14}C]inulin-loaded (50 $\mu\text{Ci}/\text{mL}$) osmotic minipumps (1007-D; Alza Corp. Palo Alto, CA) (10). Twenty-four hours later, four consecutive 24-h collections of urine were obtained. Blood was obtained (retro-orbital puncture under halothane anesthesia), bracketing the urine collections. Inulin was measured in plasma and urine by scintillation spectrometry. The clearance of inulin was used to estimate GFR (in microliters per minute).

After the collections, the animals were anesthetized (pentobarbital, 80 mg/kg ip). The abdominal thoracic cavity was opened. An 18-gauge needle was inserted into the left ventricle, and a cacodylate-buffered glutaraldehyde solution was pumped continuously at 100 to 110 mm Hg. At the start of the infusion, the inferior vena cava was incised. Approximately 60 to 80 mL of solution was infused before the left kidney was removed and weighed and the cortex was diced into 1-mm cubes. These were washed in buffer, postfixed in 1% osmium tetroxide, dehydrated in graded alcohol, and embedded in epon (10 blocks/animal). One (0.7- μm) thick section was cut per block and stained with toluidine blue. Selected glomeruli were photographed at a magnification of $\times 40$. Glomeruli were chosen by a stratified sampling technique, as described previously (10). Negatives were printed to a uniform size (magnification, $\times 1,000$). The surface density (Sv) of open glomerular capillary loops was determined by an unbiased line-intercept method on 8 to 14 glomeruli. Fractional mesangial volume (Mv) and cell density (Nv) were measured by point-counting techniques (10,13). Three to six glomeruli for each animal were examined by electron microscopy. Photomicrographs were taken at a magnification of $\times 6,000$. The number of filtration slits per micrometer of glomerular

basement membrane (GBM) length (slits per micrometer of GBM) and the percentage of the GBM occupied by osmophilic deposits were calculated (10,14). Glomerular volume (Gv) was measured on at least 50 free-floating glomeruli by a previously described method (14).

Plasma samples for anti-dsDNA and IL-6 were taken at 0, 14, and 28 days. Neutralizing antibodies to mAb IL-6R were examined at Days 0, 7, and 21. Anti-dsDNA (ELISAS USA Inc, Osceola, WI) and IL-6 (Endogen Inc, Boston, MA) were measured by commercial ELISA kits. Neutralizing antibodies were measured by a modified technique described by Tanaka *et al.* (15). Briefly, 96-well microtiter plates were coated with 20 $\mu\text{g}/\text{mL}$ of mAb IL-6R in 0.05 M carbonate-bicarbonate buffer overnight at 4°C. The plates were washed with phosphate buffered saline–0.05% Tween-20 and blocked with BSA, and serum samples (50 $\mu\text{L}/\text{well}$, 1/200 to 1/1600) were added. After overnight incubation at 4°C, the plates were washed and alkaline phosphatase–conjugated sheep anti-mouse IgG (Organon Teknika Inc, Scarborough, Ontario, Canada) was incubated for an additional 4 h. The alkaline phosphatase substrate (Sigma Chemical Co.) was added, and the reaction was stopped 30 min later with 3 N NaOH. The color change at 410 nm was read on a Microelisa Reader (MR-600A; Dynatech Lab Inc, Alexandria, VA). Mouse IgG was measured by ELISA in the urine and plasma obtained in 21-wk-old mice undergoing the GFR measurements, and the fractional excretion of IgG (θIgG) was calculated (14).

Data are presented as mean \pm SD. A *t* test was used to determine whether significant differences occurred between controls and the two diseased mouse groups (IgG and REC). A *P* value of $<5\%$ was considered statistically significant.

TABLE 1. Glomerular function and structure

	CON (<i>N</i> = 6)	REC (<i>N</i> = 5)	IgG (<i>N</i> = 5)
GFR ($\mu\text{L}/\text{min}$)	285 \pm 63	254 \pm 61	165 \pm 76 ^b
θIgG ($\times 10^{-5}$)	0.1 \pm 0.1	0.8 \pm 0.4 ^c	1.4 \pm 1.4 ^c
GV ($\mu\text{m}^3 \times 10^3$)	292 \pm 24	519 \pm 79 ^c	529 \pm 119 ^d
Mv ($\mu\text{m}^3/\mu\text{m}^3$)	0.19 \pm 0.04	0.21 \pm 0.04	0.40 \pm 0.04 ^d
Sv ($\mu\text{m}/\mu\text{m}^2$)	0.20 \pm 0.01	0.18 \pm 0.01	0.13 \pm 0.04 ^c
FSA ($\mu\text{m}^2 \times 10^3$)	59 \pm 17	95 \pm 15 ^c	66 \pm 11
Nv (nuclei/ $\mu\text{m}^3 \times 10^{-3}$)	1.23 \pm 0.21	1.21 \pm 0.08	1.39 \pm 0.17
DD (%)	0.0 \pm 0.0	3.1 \pm 3.2	2.9 \pm 3.5
Slits/ μm of GBM	2.58 \pm 0.14	2.01 \pm 0.10 ^d	1.62 \pm 0.55 ^c

^a θIgG , fractional excretion of IgG; FSA, filtration surface area; DD, percentage of GBM occupied by dense deposits; slits/ μm of GBM, number of filtration slits per micrometer of GBM.

^b *P* < 0.05, CON versus IgG.

^c *P* < 0.01, CON versus REC and IgG.

^d *P* < 0.001, CON versus REC and IgG.

RESULTS

One animal in each of the REC and IgG groups died before the final measurements and was excluded from the structure and function analysis. Compared with CON mice, GFR was reduced in IgG but not in REC-treated mice (Table 1). Glomerular capillary wall size permeability, measured as the fractional excretion of IgG (θ IgG), was abnormal in both diseased groups. Figure 1 displays typical glomeruli from animals of each group. Consistent with the morphometric measurements in Table 1, glomeruli from REC mice looked essentially normal, but were enlarged. Glomeruli from IgG-treated mice were hypertrophied and demonstrated a doubling of Mv. The Sv of open capillary loops was reduced in the rat IgG-treated group, but not in mAb IL-6R-treated mice. Filtration surface area ($Gv \times Sv$) per glomerulus was greatly elevated in REC mice compared with both IgG and CON animals. Nv tended to be higher in IgG mice. Cells per glomerulus ($Nv \times GV$) were increased in both diseased groups compared with CON animals (727 ± 139 and 628 ± 119 versus 360 ± 59 ; IgG and REC versus CON; $P < 0.01$).

Anti-dsDNA antibodies were elevated at the start of treatment in the 15-wk-old MRL-lpr/lpr mice (114 ± 76 and 60 ± 18 IU/mL, REC and IgG, respectively) but were not detected in the MRL-+/+ mice. At Week 2, anti-dsDNA antibodies fell to undetectable levels in five of the six REC mice. Autoantibody levels were significantly lower in REC mice, on average, compared with IgG mice (18 ± 37 versus 71 ± 26 IU/mL; $P < 0.05$). By Week 4 the reverse occurred; anti-dsDNA antibodies were elevated to a greater extent in REC mice (229 ± 107 versus 88 ± 23 IU/mL, REC versus IgG; $P < 0.05$). IL-6 levels were high in both IgG and REC mice (151 ± 160 and 240 ± 92 pg/mL, respectively) compared with those in CON animals (25 ± 34 pg/mL; $P < 0.01$) at age 15 wk and increased progressively in REC and IgG mice (955 ± 290 and 472 ± 280 , respectively). High titer neutralizing antibodies ($>1/1600$) to the mAb IL-6R were detected by Week 3 in all REC animals. No neutralizing antibodies were detected in any animal before the start of treatment.

DISCUSSION

mAb therapy against the IL-6R has favorable effects on renal function (GFR) and structure in the MRL-lpr/lpr murine model of SLE. Treatment reduced Mv dramatically. Favorable effects on cell proliferation, Gv, and proteinuria (as evaluated by θ IgG) were not evident. The effect of IL-6R blockage on anti-dsDNA antibody levels *in vivo* was also remarkable but was short-lived. It is difficult to determine whether more-favorable effects would have resulted

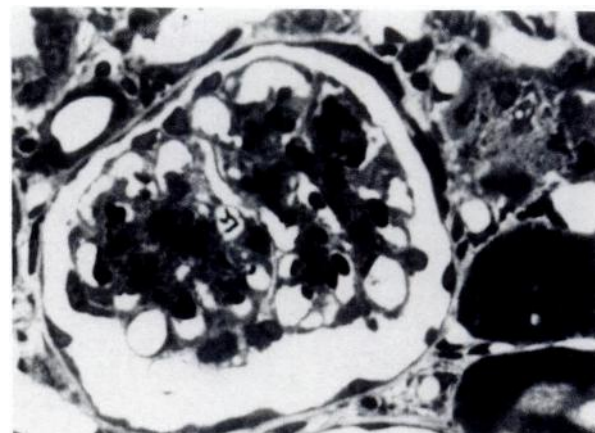
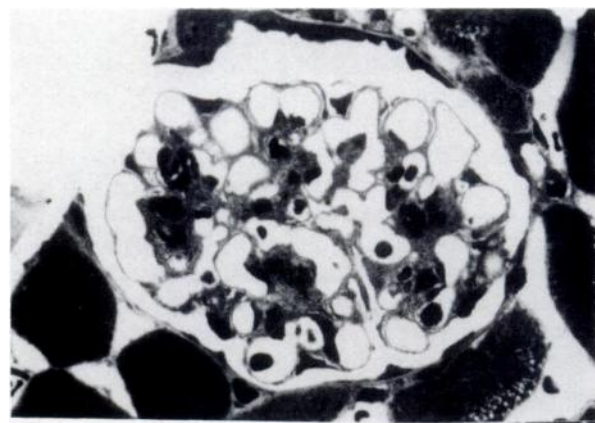
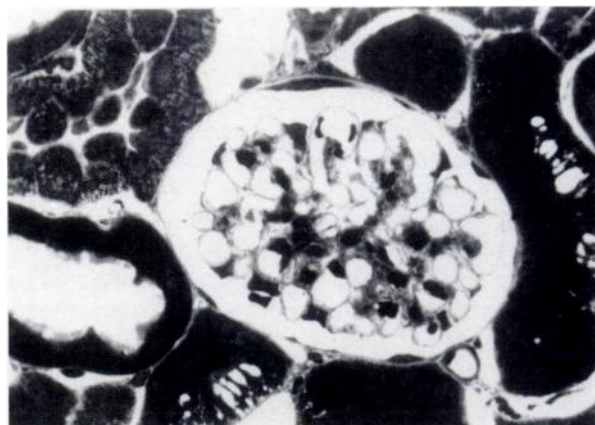


Figure 1. Glomerular structure in 21-wk-old mice (magnification, approximately $\times 250$). Top, normal glomerulus from +/+ animals. Middle, glomerular enlargement but near-normal Mv from a REC-treated mouse. Bottom, both glomerular hypertrophy and mesangial matrix expansion are demonstrated in an IgG-treated mouse.

if neutralizing antibodies and/or the progressive increase in plasma IL-6 failed to occur.

One criticism of the study centers around the use of an appropriate control group. Ideally, an irrelevant mAb should have been used, but any foreign protein might aggravate the renal injury in the MRL-lpr/lpr mouse. Previous work in this laboratory in untreated MRL-lpr/lpr mice has shown that 14-wk-old animals have normal function and structure by light and electron microscopy (10). GFR is $257 \pm 43 \mu\text{L}/\text{min}$ ($N = 15$), and Mv is 0.21 ± 0.06 . By age 20 wk, GFR falls to $178 \pm 50 \mu\text{L}/\text{min}$ ($N = 17$), whereas Mv increases to 0.33 ± 0.07 . It is possible the rat IgG may have aggravated the injury because function and structure were worse compared with these historical controls. Nonetheless, the mAb IL-6R appeared to preserve function and limit the increase in Mv compared even with these animals.

The source of the elevated IL-6 in this model could be "locally" generated by injured resident glomerular cells or "systemically" produced by the null T lymphocytes proliferating throughout the body (16,17). Autoreactive T lymphocytes accumulate presumably because of a defective *fas* gene that prevents programmed cell death (18). These cells have been shown to have increased levels of IL-6 mRNA (17,19). Whatever the source, this study demonstrates that IL-6 inhibition could prove to be a useful adjunctive treatment in lupus nephritis.

ACKNOWLEDGMENTS

This work was funded by the Medical Research Council of Canada and The National Kidney Foundation of Canada. Technical assistance was provided by Elizabeth Steeves and Karin Lavin. Many thanks to Heather Fewtrell for providing the secretarial assistance.

REFERENCES

- Balow JE, Austin HA II, Tsokos GC, *et al.*: Lupus nephritis. *Ann Intern Med* 1987;106:79-94.
- Steinberg AD: The treatment of lupus nephritis. *Kidney Int* 1986;30:769-787.
- Hirano T, Akira S, Taga T, Kishimoto T: Biological and clinical aspects of interleukin-6. *Immunol Today* 1990;11:443-449.
- Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR: Elevated levels of endogenous IL-6 in systemic lupus erythematosus. *J Immunol* 1991;147:117-123.
- Kitani A, Hara M, Hirose T, *et al.*: Autostimulatory effects of IL-6 on excessive B cell differentiation in patients with systemic lupus erythematosus: Analysis of IL-6 production and IL-6R expression. *Clin Exp Immunol* 1992;88:75-83.
- Ruef C, Budde K, Lacy J, *et al.*: Interleukin 6 is an autocrine growth factor for mesangial cells. *Kidney Int* 1990;38:249-257.
- Horii Y, Muraguchi A, Iwano M, *et al.*: Involvement of IL-6 in mesangial proliferative glomerulonephritis. *J Immunol* 1989;143:3949-3955.
- Suematsu S, Matsuda T, Aozasa K, *et al.*: IgG1 plasmacytosis in interleukin 6 transgenic mice. *Proc Natl Acad Sci USA* 1989;86:7547-7551.
- Kiberd BA: Interleukin 6 in murine lupus nephritis [Abstract]. *J Am Soc Nephrol* 1992;3:598.
- Kiberd BA: The functional and structural changes of the glomerulus throughout the course of murine lupus nephritis. *J Am Soc Nephrol* 1992;3:930-939.
- Vink A, Coulie P, Warnier G, *et al.*: Mouse plasmacytoma growth in vivo: Enhancement by interleukin-6 and inhibition by antibodies directed against IL-6 or its receptor. *J Exp Med* 1990;172:997-1000.
- Coulie PG, Vink A, VanSnick J: A monoclonal antibody specific for the murine IL-6 receptor inhibits the growth of a mouse plasmacytoma in vivo. *Curr Topics Microbiol Immunol* 1990;466:43-46.
- Miller PL, Meyer TW: Effects of tissue preparation on glomerular volume and capillary structure in the rat. *Lab Invest* 1990;63:862-866.
- Kiberd BA: Murine lupus nephritis: A structure function study. *Lab Invest* 1991;65:51-60.
- Tanaka K, Hancock WW, Osawa H, *et al.*: Mechanism of action of anti-IL-2R monoclonal antibodies. *J Immunol* 1989;143:2873-2879.
- Iwano M, Dohi K, Hirata E, Horii Y, Shiiki H, Ishikawa H: Induction of interleukin 6 synthesis in mouse glomeruli and cultured mesangial cells. *Nephron* 1992;62:58-65.
- Gallo CD, Jevnikar AM, Brennan DC, Florquin S, Pacheco-Silva A, Kelley VR: Autoreactive kidney-infiltrating T-cell clones in murine lupus nephritis. *Kidney Int* 1992;42:851-859.
- Watanabe-Fukunaga R, Brannan CI, Copeland NG, Jenkins NA, Nagata S: Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature (Lond)* 1992;356:314-317.
- Murray LJ, Lee R, Martens C: In vivo cytokine gene expression in T-cell subjects at the autoimmune MRL/MP-lpr/lpr mouse. *Eur J Immunol* 1990;20:163-170.