Modulation of Neutrophil Influx in Glomerulonephritis in the Rat with Anti–Macrophage Inflammatory Protein-2 (MIP-2) Antibody

Lili Feng, Yiyang Xia, Teizo Yoshimura,* and Curtis B. Wilson

*Department of Immunology, The Scripps Research Institute, La Jolla, California 92037; and *Immunopathology Section, Laboratory of Immunobiology, National Cancer Institute, Frederick Cancer Research & Development Center, Frederick, Maryland 21702*

Abstract

The role of the chemokine, macrophage inflammatory protein-2 (MIP-2), during anti–glomerular basement membrane (GBM) antibody (Ab) glomerulonephritis (GN) was studied. Rat MIP-2 cDNA had been cloned previously. Recombinant rat MIP-2 (rMIP-2) from *Escherichia coli* exhibited neutrophil chemotactic activity and produced neutrophil influx when injected into the rat bladder wall. By using a riboprobe derived from the cDNA and an anti–rMIP-2 polyclonal Ab, MIP-2 was found to be induced in glomeruli with anti-GBM Ab GN as mRNA by 30 min and protein by 4 h, with both disappearing by 24 h. The expression of MIP-2 correlated with glomerular neutrophil influx. A single dose of the anti–MIP-2 Ab 30 min before anti-GBM Ab was effective in reducing neutrophil influx (40% at 4 h, $P < 0.01$) and periodic acid-Schiff deposits containing fibrin (54% at 24 h, $P < 0.01$). The anti–rMIP-2 Ab had no effect on anti-GBM Ab binding (paired-label isotope study). Functional improvement in the glomerular damage was evidenced by a reduction of abnormal proteinuria ($P < 0.05$). These results suggest that MIP-2 is a major neutrophil chemoattractant contributing to influx of neutrophils in Ab-induced glomerular inflammation in the rat. (*J. Clin. Invest.* 1995. 95:1009–1017.) Key words: macrophage inflammatory protein-2 • anti–glomerular basement membrane glomerulonephritis • anti–macrophage inflammatory protein-2 antibody • neutrophils • chemokine

Introduction

Neutrophil migration from blood into tissue depends on a cascade of events including chemoattractant activation and selectins and integrin-related adhesion of cells to the endothelium (1–6). Numerous chemoattractants are involved in this process including leukotrienes, complement components, and the recently described chemokine superfamily. This superfamily of basic, heparin-binding molecules of molecular mass 8–10 kD with chemotactic and inflammatory properties has been termed intercrines, small cytokines, and recently by the recommended nomenclature, chemokines (7). The superfamily is characterized by four conserved cysteines with two disulfide bridges and is subdivided into the α subfamily, in which the first two cysteines are separated by another amino acid (C-X-C), and the β subfamily, in which the cysteines are adjacent (C-C) (for reviews see references 8 and 9). The α subfamily includes platelet factor-4 (PF-4), interleukin-8 (IL-8/NAP-1), GRO/MGSA, cytokine-induced neutrophil chemoattractant (CINC)[1]/KC, and macrophage inflammatory protein-2 (MIP-2), which are neutrophil chemoattractants and activators. The β subfamily includes RANTES, MIP-1, and monocyte chemotactic protein-1 (MCP-1)/JE. The two subfamilies represent two gene clusters on two different chromosomes.

In the rat, IL-8, a major neutrophil chemoattractant in humans, has not been identified with certainty, and CINC/KC and MIP-2 may provide this function. These two chemokines are presumed to be homologues of the human GROα, β, and γ genes (10–13). CINC/KC was isolated from the rat kidney epithelioid cell line NRK-52E and has been cloned (14, 15). Previous studies in the mouse showed that anti–MIP-2 antibody (Ab) delayed, but did not prevent, inflammation produced by intracisternal challenge of pneumococci (16); however, it did reduce by $\sim 40\%$ the influx of neutrophils in a model of *Mycobacterium bovis bacilli Calmette-Guérin* (BCG)-induced peritonitis (17). Pulmonary instillation of lipopolysaccharide (LPS) in rats produced a rapid and marked increase in MIP-2 and KC levels (18), and a marked induction of both KC and MIP-2 was observed in the alveolar macrophages of hamsters after injection of opsonized particles (19).

To determine the role of MIP-2 in inflammatory glomerular injury, we used a rat MIP-2 cDNA recovered previously during cloning of rat *gro*/KC and expressed recombinant MIP-2 (rMIP-2). The rMIP-2 attracted neutrophils in a chemotaxis assay and produced neutrophil influx when injected into the rat bladder wall; the rMIP-2 also was able to attract neutrophil influx into rat lung by intratracheal instillation (Rose, C. E., personal communication). We then evaluated the protective effects of a polyclonal Ab raised to rat rMIP-2 in experimental anti–glomerular basement membrane (GBM) Ab-induced glomerulonephritis (GN).

Methods

Construction of a cDNA library, screening, and DNA sequencing. Spleen cells obtained from Fisher rats were incubated with 5 μg/ml of Con A for 4 h, poly (A) RNA was isolated, and cDNA synthesized by

Address correspondence to Curtis B. Wilson, MD, Department of Immunology (IMM5), The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037. Phone: 619-554-8056; FAX: 619-554-6705.

1. *Abbreviations used in this paper:* CINC, cytokine-induced neutrophil chemoattractant; GAP, riboprobe for GAPDH; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBM, glomerular basement membrane; GN, glomerulonephritis; ICAM-1, intercellular adhesion molecule-1; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; NRS, normal rabbit serum; rMIP-2, recombinant MIP-2.

a modification of the Gubler and Hoffman method (20) was used to prepare a library in λ ZAP II vector.

Oligodeoxynucleotide probes were prepared from a portion of human GRO cDNA sequence, where a highly conserved sequence was expected based on the similarity between human and mouse GRO (10). The sequence of the probe was 5'-dGTG GCT ATG ATC TCG GTT TGG GTG CAG TGG-3'. Approximately 5×10^5 recombinant phages from the cDNA library were screened by high-density plaque hybridization with the ^{32}P-labeled oligonucleotide probe. Phagemids carried within λ ZAP II recombinants were rescued with helper phage (21). cDNA inserts were sequenced by the dideoxynucleoside triphosphate chain termination method (22).

Expression of rat MIP-2 in Escherichia coli. DNA encoding MIP-2 without signal peptide was PCR-amplified and subcloned into pET-M1 (23). pET-M1 was modified from pET-11a with a sequence coding for 6 × histidine residues (6 × His) for ease in purification (23, 24). The host strain, BL21 (DE3), was transformed by pETMIP-2 and was cultured in LB broth with ampicillin (100 μg/ml) and induced with 0.5 mM of isopropyl-β-thiogalactopyranoside at 37°C for 4 h. The cell culture was centrifuged at 5,000 g for 10 min, and the pellet was stored at −20°C for subsequent purification.

An Ni-NTA affinity resin for binding the 6 × His tag was purchased from Invitrogen (San Diego, CA). The purification procedure of the manufacturer was used with some modifications, as described previously (24).

Purification and refolding of rMIP-2 and Ab production. The purified, denatured, and insoluble rMIP-2 was dialyzed against reduction buffer (100 mM Tris-HCl [pH 7.5] and 4 M urea) at the concentration of 1 mg/ml. To reduce the concentration of the denaturing reagents and to initiate refolding, this mixture was diluted at 4°C with dilution buffer (100 mM Tris-HCl [pH 8.0], 100 mM NaCl, 5 mM CaCl₂ and 0.01% Triton X-100) to a final concentration of 0.1 M urea. The dilution step was performed by adding 10 ml/h dilution buffer while gently stirring, thus allowing reshuffling of the mismatched disulfide bonds. After refolding, buffer was exchanged to 0.9% NaCl by dialysis.

A polyclonal antiserum was raised by immunizing a rabbit with rMIP-2. An initial dose of 1 mg of rMIP-2 with complete Freund's adjuvant was injected subcutaneously. Subsequent doses of 0.5 mg in incomplete Freund's adjuvant were given weekly.

Assessment of neutrophil chemotaxis. Rat peritoneal neutrophils were prepared by injection of 5 ml of 0.2% sodium caseinate in phosphate-buffered saline into the peritoneal cavities of Fisher rats (25). The neutrophil chemotaxis assay was performed with a 10-μM-thick polyvinylpyrrolidone-free 3-μM pore size polycarbonate filter (26). The number of neutrophils per well (in triplicate) was 5×10^4. After 40 min at 37°C in humidified air with 5% CO₂, filters were removed. Cells that did not migrate were wiped away; the filters were air dried and stained with Diff-Quik (American Scientific Products, McGaw Park, IL). Migrated cells were counted microscopically at a magnification of 400. HBSS was the negative control. The experiment has been repeated three times.

To assess in vivo chemotactic activity, either 50 μl of a rMIP-2 solution (200 μg/ml) or phosphate-buffered saline was injected into the bladder wall of anesthetized Lewis rats under direct vision using a 30-gauge needle. The bladders were obtained for histologic evaluation 2 h later.

Transfection of rat MIP-2 into COS-7. The full-length rat MIP-2 was subcloned into pCDM8 (Invitrogen) to generate pCDM8MIP-2. pCDM8MIP-2 was transfected into subconfluent COS-7 by electroporation of 8×10^6/ml cells at 1,500 V, 1,000 μF. The antisense clone in the same vector was used for mock transfection as a control. After 48 h of incubation with serum-free medium, the supernatant was collected for chemoattractant assay and immunoblot. The cell pellet was used for RNA extraction.

Anti-GBM Ab GN. Anti-GBM Ab-induced GN was produced as previously published using a rabbit anti–rat GBM Ab that had been decomplemented and absorbed with rat peripheral blood cells (27). Four groups of six rats each were given either 0.5 ml of anti–MIP-

2 Ab or normal rabbit serum (NRS) intravenously 30 min prior to administration of anti-GBM Ab or normal rabbit serum (NRS). Control and anti–MIP-2 Ab groups ($n = 6$) were examined at 4 or 24 h for glomerular neutrophil influx and 24-h proteinuria. To assess the effects of the anti–MIP-2 Ab on binding of anti-GBM Ab, a paired label isotopic binding assay was done as described previously (28).

Probe preparation, glomerular RNA isolation, and RNase protection assay. For antisense riboprobe synthesis, the rat MIP-2 (1–174) was used for in vitro transcription. cDNAs of rat TNFα (818–1144; GenBank accession No. L00981) and IL-1β (450–667; GenBank accession No. M98820) were used for preparation of riboprobes. Rat intercellular adhesion molecule-1 (ICAM-1) (24) was described previously (29, 30). Glomeruli were isolated as before (29). After washing with 0.9% saline, the glomeruli were homogenized in 4 M guanidine isothiocyante with a sonicator (Heat Systems-Ultrasonics, Plainview, NY). The RNA was prepared by a single-step method (31). For study, 5 μg of glomerular total RNA was hybridized with 1×10^5 cpm of each [α^{32}P] rat antisense riboprobes for MIP-2, ICAM-1, TNFα, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase (GAP), which has been described previously (24). The RNase protection assay was described previously (24), with the protected bands quantitated by a radioanalytic imaging system (AMBIS Systems, San Diego, CA). The data are presented as a ratio of the counts per minute for specific mRNA/GAPDH mRNA bands to ensure a constant quantity of RNA in each sample.

Western blot. The level of MIP-2 was analyzed by Western blot analysis as described previously (26, 32). In brief, glomeruli from the three rats at each time point were pooled per preparation. The glomeruli were homogenized in phosphate-buffered saline with protease inhibitors. After centrifugation, the supernatant was collected. The supernatant was incubated with protein A-Sepharose 4B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) for 1 h to remove rabbit Ig. After centrifugation, the supernatant was quantitated for protein content by the Bradford assay, 100 μg of each sample was electrophoresed on SDS-polyacrylamide gel and transferred onto nitrocellulose (Schleicher & Schuell, Inc., Keene, NH). The supernatant from transfectants was used directly for loading without further treatment. The blot was incubated in the Ab solution (diluted 1:250) overnight and was treated with goat anti–rabbit IgG conjugated with alkaline phosphatase (diluted 1:1,500).

Morphologic study. At time of killing, renal tissue samples were fixed in 10% formalin for light microscopy or were snap-frozen in liquid nitrogen with Tissue-Tek (Miles, Inc., Elkhart, IN) for immunofluorescence microscopy. Histologic examination was performed on paraffin sections (2–3 μm) stained with periodic acid-Schiff reaction and hematoxylin counterstain. Glomerular neutrophil influx was determined by counting neutrophils in 35 paraffin-embedded glomerular cross-sections of kidney stained with periodic acid-Schiff (27). All morphologic and functional data are presented as mean±SE. Statistical analysis was done using the Mann-Whitney test.

In situ hybridization. In situ hybridization was performed as described previously with some modification (33). Briefly, kidneys were perfused and prefixed with 4% paraformaldehyde. The tissues were embedded in OCT (Miles, Inc.) and then frozen on dry ice. ^{35}S-Labeled antisense and sense riboprobes were transcribed from both directions using MIP-2 cDNA cloned in pBluescript. Frozen sections (4–6 μm thick) were fixed in 4% paraformaldehyde for 10 min on ice and deproteinated in proteinase K solution (1 μg/ml) for 10 min at room temperature. After washing in 0.5 × SSC for 10 min, prehybridization was performed at 42°C for 2 h in 50 μl of prehybridization buffer per section. Prehybridization buffer contained 10 mM DTT, 0.3 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 1 × Denhardt's, 10% dextran sulfate, 50% formamide. 10 μl of hybridization buffer with radiolabeled probe (3×10^5 cpm per section) was added directly into the bubble of prehybridization buffer covering the sections. Hybridization was performed at 55°C overnight. After washing in 2 × SSC, slides were immersed in RNase digestion buffer (20 μg/ml RNase A) at room temperature for 30 min. The slides were then washed sequentially in 2 × SSC with 2 mM EDTA, 0.1 × SSC with 1 mM EDTA, and 10 mM β-mercaptoethanol at 50°C for 2 h. The slides were finally washed in 0.5 × SSC without

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                                    -25 TCCAACCACTCTTTGGTCCAGAGCC -1

ATG GCC CCT CCC ACT CGC CAG CTC CTC AAT GCT GTA CTG GTC CTG CTC  48
MET Ala Pro Pro Thr Arg Gln Leu Leu Asn Ala Val Leu Val Leu Leu  16
                                                  *
CTC CTG CTG GCC ACC AAC CAT CAG GGT ACA GGG GTT GTT GTG GCC AGT  96
Leu Leu Leu Ala Thr Asn His Gln Gly Thr Gly Val Val Val Ala Ser  32

GAG CTG CGC TGT CAA TGC CTG ACG ACC CTA CCA AGG GTT GAC TTC AAG 144
Glu Leu Arg Cys Gln Cys Leu Thr Thr Leu Pro Arg Val Asp Phe Lys  48

AAC ATC CAG AGC TTG ACG GTG ACC CCT CCA GGA CCC CAC TGC GCC CAG 192
Asn Ile Gln Ser Leu Thr Val Thr Pro Pro Gly Pro His Cys Ala Gln  64

ACA GAA GTC ATA GCC ACT CTT AAG GAT GGT CAT GAA GTT TGT CTC AAC 240
Thr Glu Val Ile Ala Thr Leu Lys Asp Gly His Glu Val Cys Leu Asn  80

CCT GAA GCC CCC TTG GTT CAG AGG ATC GTC CAA AAG ATA CTG AAC AAA 288
Pro Glu Ala Pro Leu Val Gln Arg Ile Val Gln Lys Ile Leu Asn Lys  96

GGC AAG GCT AAC TGA CCTGGGAAGGAAGAACATGGGCTCCTGTACCC            335
Gly Lys Ala Asn END                                            100
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Figure 1. The nucleotide (*upper case*) and deduced amino acid (*lower case*) sequences of rat MIP-2 are shown. The cleavage site of the signal peptide is marked with an asterisk. The conserved cysteines are shadowed.

EDTA and β-mercaptoethanol for 10 min twice. After dehydration, the slides were dried. After autoradiography for 3 d, the slides were dipped in Kodak NTB2 nuclear emulsion diluted in 1:1 with water at 42°C. Exposure was done at 4°C in the dark for 4 wk before development.

Results

Cloning of rat MIP-2. About 5 × 10^5 phages were initially screened with the *gro* oligonucleotide probe shown in Methods. After two rounds of screening and DNA sequencing from denatured plasmids, it was found that one clone encoded for rat MIP-2 (Fig. 1). Rat MIP-2 cDNA had 1,008 bp with an open reading frame of 300 bp, which encodes for a 100–amino acid protein. The cDNA clone did not contain a poly (A) tail. The sequence was identical to a partial rat MIP-2 cDNA published by Huang et al. (18). The deduced amino acid sequence of rat MIP-2 was highly similar to that of mouse MIP-2 (10). As reported by Tekamp-Olson et al. (10), human homologues of mouse MIP-2 are identical to human GROβ and GROγ. In the 3' untranslated region, there were 10 copies of AU-rich motifs, which are involved in the regulation of mRNA stability (34).

E. coli expression of rMIP-2 and chemotactic assay in vitro and in vivo. The rMIP-2 was expressed as a fusion protein with a 6 × His tag at the amino termini in BL21 (DE3) under the control of T7 RNA polymerase. The expected molecular mass of the MIP-2 peptide (amino acids 29–100) is 8 kD; the molecular mass of the peptide coded by polylinker region and 6 × His is 0.3 kD. The size of the total molecule is ~ 8.3 kD. Fig. 2 is a Western blot which shows the two forms of MIP-2 expressed in COS-7 (lane *3*) and in *E. coli* (lane *4*) that reacted with the Ab generated against the rMIP-2. The higher molecular weight of the *E. coli* form (lane *4*) is due to the extra amino acids fused with MIP-2 during expression. The rMIP-2 expressed in *E. coli* was purified and showed chemotactic activity detected as the number of cells that migrated through the filter, with the activity shown at the dose of 40 ng/ml, while the peak was at the dose of 1 μg/ml. Fig. 3 shows the chemotactic activity of rat rMIP-2 versus rat peritoneal neutrophils. Margination and extravasation of neutrophils was induced mainly in and around venules in the lamina propria of the rat bladder 2 h after administration of rMIP-2 as shown in Fig. 4. Intratracheal instillation of rMIP-2 caused a dramatic increase in neutrophil influx (Rose, C. E., personal communication).
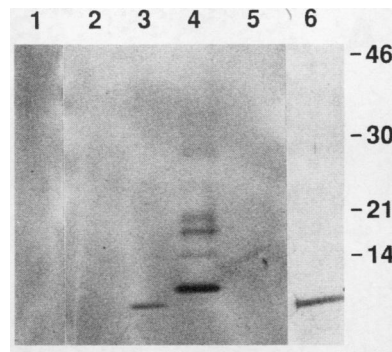

Figure 2. The specificity of the anti–MIP-2 Ab and expression of MIP-2 recombinant protein, purification by Ni-NTA affinity resin, and immunoblot of KC polypeptide are shown as described in Methods. Preimmune serum showed no reactivity (lane *1*) with 20 μl of the supernatant from COS-7 transfected with sense MIP-2 in pCDM8. No reaction was seen (lane *2*) with 20 μl of supernatant from COS-7 transfected with antisense MIP-2 in pCDM8 when reacted with the anti–MIP-2 Ab. In lane *3*, the anti–MIP-2 Ab reacted with the same supernatant from the sense MIP-2 transfected COS-7 used in lane *1*. In lane *4*, the rMIP-2 expressed in *E. coli* reacted with the anti–MIP-2 Ab. In lane *5*, the anti–MIP-2 Ab had no reactivity with the serum-free supernatant of a KC transfectant, whereas this KC-containing supernatant was reactive with anti-KC antibody, as shown in lane *6*. Numbers on the right are kilodaltons.

Specificity of anti–rMIP-2 Ab. The antiserum generated against rMIP-2 reacted monospecifically against supernatant from COS-7 transfectants with rat MIP-2, pCDM8MIP-2, but did not recognize any proteins in supernatants from antisense transfectants in the same plasmid (Fig. 2, lane *2*). Preimmune serum also showed no reactivity (Fig. 2, lane *1*). In particular, antiserum to MIP-2 did not cross-react with the serum-free supernatant from KC transfectants (lane *5*), while anti-KC did react (lane *6*).

Expression of MIP-2 in the glomeruli by RNase protection assay and in situ hybridization. mRNA expression of MIP-2 in glomeruli was as early as 30 min after Ab injection (data not shown). As shown in Fig. 5, at the 4-h time point the mRNA
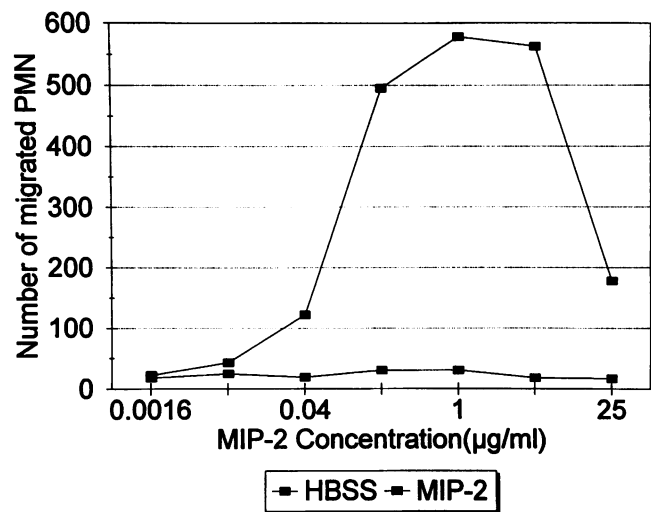

Figure 3. The in vitro chemotactic activity of the rMIP-2 is shown for one study which is representative of three done. The data are presented as the mean of triplicate wells for the numbers of PMNs that migrated toward the increasing concentrations of rMIP-2 in comparison with a HBSS with 0.2% BSA as a negative control. The triplicate wells were in close agreement.
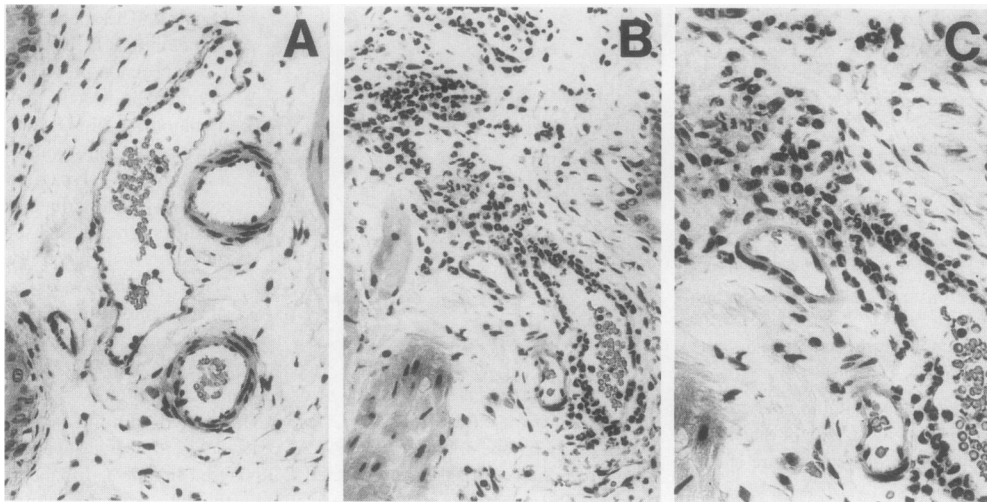
Figure 4. The injection of the bladder wall with saline (*A*) caused no influx in neutrophils in contrast to *r*MIP-2, which caused margination and extravasation of neutrophils in and around the small venules at low and high power (*B* and *C*).

level was still positive, which correlated well with the neutrophil infiltration; at 24 h, it declined to the level not detectable. Interestingly, treatment with anti–MIP-2 Ab affected the mRNA level of MIP-2. At 4 h, the MIP-2 mRNA level of the group II (anti-GBM Ab and 0.5 ml of anti–MIP-2) was $\sim$ 1.35±0.493, as scanned by AMBIS and factored relatively by GAP, significantly lower than 2.675±0.532 ($P < 0.05$), the level of group I (anti-GBM Ab and 0.5 ml of NRS). The attenuation of inflammatory infiltrates by the anti–MIP-2 Ab might account for this reduction. MIP-2 protein expressed in the glomeruli was detected by Western blot (Fig. 5). MIP-2 protein expression correlated with the mRNA level expressed at 4 h and declined at 24 h. There was no marked difference of protein level between group I and group II as shown at mRNA level (Fig. 6), perhaps due to the difference of the sensitivity between two methods.

In situ hybridization demonstrated that the MIP-2 mRNA was confined to the glomeruli 4 h after anti-GBM Ab administration (Fig. 7). The individual cells responsible for the mRNA included mesangial cells and intraluminal inflammatory cells.

mRNA expression of ICAM-1, IL-1β, and TNFα in the glomeruli. The time course of mRNA expression of ICAM-1, IL-1β, and TNFα during GN was similar to that found before

(29, 30). Anti–MIP-2 Ab treatment did not change the mRNA expression levels of these three genes according to AMBIS-scanned and quantitated data which were factored relative to the GAPDH mRNA levels as shown in Fig. 8. These findings suggest that the anti–MIP-2 Ab did not have any overall effect on the underlying inflammatory mediator stimulus for the generation of the anti-GBM Ab-induced lesion.

Histology. The glomerular histologic features were those of a severe infiltrative/proliferative GN with large numbers of infiltrating neutrophils at 4 h (Fig. 9 *A*), which largely disappeared by 24 h. The mean of 608±26 neutrophils ($n = 6$) per 35 glomeruli (17.4 neutrophils/glomerulus) in the NRS control group was reduced by $\sim$ 40% to 362±6 ($n = 6$) (10.4 neutrophils/glomerulus) with anti–MIP-2 Ab administration ($P < 0.01$) (Fig. 9 *B*). The anti–MIP-2 Ab had its greatest effect in reducing infiltration of high numbers of neutrophils, so that only 18.1% of glomeruli had 15 or more neutrophils per glomerulus after anti–MIP-2 Ab treatment compared with 64.7% in the NRS control group. By 24 h the neutrophils had largely disappeared, as is the usual finding in this model, with 54±3 compared with 35±2 per 35 glomeruli ($P < 0.01$) (1.5 vs 1.0 per glomerulus) in the NRS control and anti–MIP-2 Ab groups ($n = 6$ each), respectively. Mononuclear infiltrative cells were
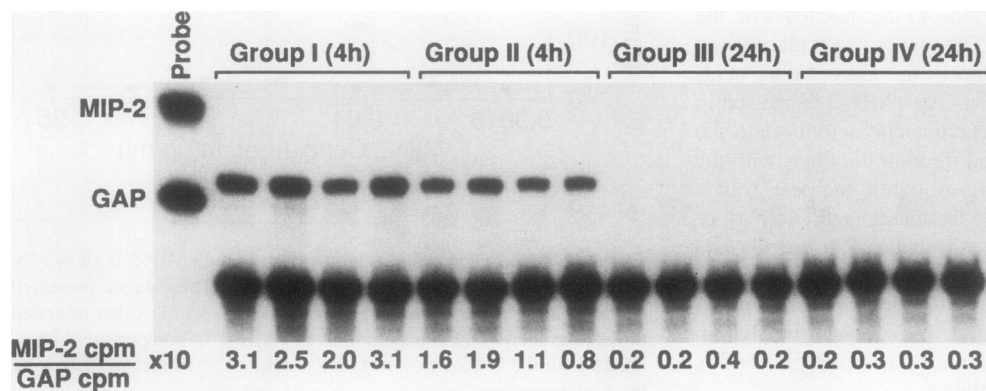

Figure 5. Expression of MIP-2 mRNA analyzed by RNase protection assay. 5 μg of total RNA was hybridized with riboprobes, as described in Methods. The [α³²P]UTP-labeled MIP-2 and GAP riboprobes were protected with the complementary mRNA from glomeruli. The gel was scanned on AMBIS, and the final values were factored relative to the GAPDH mRNA levels. In the RNase protection assay, the protected band is shorter than the probe since the polylinker regions present in the probe are not protected. Groups I and III received NRS; groups II and IV received anti–MIP-2 Ab.

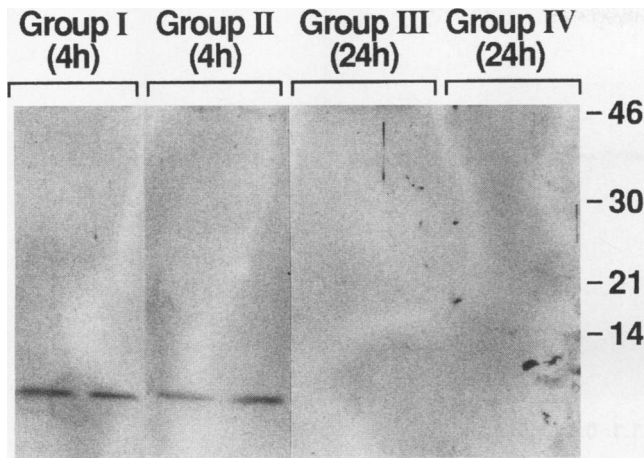|	Probe	Group I (4h)				Group II (4h)				Group III (24h)				Group IV (24h)			
MIP-2																	
GAP																	
$\dfrac{\text{MIP-2 cpm}}{\text{GAP cpm}}$ x10		3.1	2.5	2.0	3.1	1.6	1.9	1.1	0.8	0.2	0.2	0.4	0.2	0.2	0.3	0.3	0.3

Figure 6. Expression of MIP-2 protein in glomeruli analyzed by Western blot. 100 μg of total glomerular lysate from a pool of three rats was loaded.

also present. There was evidence of cell death at 24 h, with scattered nuclear debris. The histologic lesion at 24 h in the control group had large areas of periodic acid-Schiff–positive material occluding the lumens of glomerular capillaries in a mean of 65±2% of the 35 glomeruli per rat evaluated ($n = 6$) (Fig. 9 C). This material corresponded to areas of fibrin deposit detected by immunofluorescence study (29). The amount of this material was reduced by the anti–MIP-2 Ab administration, so that only a mean of 28±3% of glomeruli ($n = 6$) had any of the material detected (Fig. 9 D) ($P < 0.01$).

Renal function. Functional changes as monitored by 24-

h protein excretion were also improved after anti–MIP-2 Ab administration. The mean proteinuria of 193±16 mg/24 h in the NRS group ($n = 6$) decreased to 121±22 mg/24 h after Ab treatment ($n = 6$) ($P < 0.05$).

Binding of anti-GBM Ab and complement fixation. To determine if the beneficial effect of the anti–MIP-2 Ab could have been mediated via an inhibition of binding of the anti-GBM Ab, thereby lessening the immunonephritogenic stimulus, a paired-label binding study was done. Of the 100 μg of anti-GBM Ab given to groups of rats ($n = 3$) having received either anti–MIP-2 or NRS 30 min earlier, an identical mean of 0.381 μl Ab/g kidney was found bound in the washed renal homogenate at 24 h, indicating no interference with anti-GBM Ab binding. By immunofluorescence, no differences were detected in C3 deposits in the GBM between the groups, suggesting that the anti–MIP-2 Ab had no substantial effect on complement fixation.

Discussion

Leukocyte accumulation in vessels including the glomerulus is the end result of a cascade of events resulting from immune activation of complement and other chemoattractants including the chemokines, cytokine activation, and engagement of a number of cell adhesion molecules including the selectins and integrins with their counterreceptor molecules (35–41). For example, in the kidney glomerular endothelial cells were used to demonstrate a role for L-selectin in leukocyte binding (42). Adhesion of neutrophils and monocytes/macrophages to human mesangial cells involved ICAM-1 and CD11/CD18 (43). ICAM-1 and vascular cell adhesion molecule-1 are expressed on glomerular epithelial cells (44). ICAM-1 expression is
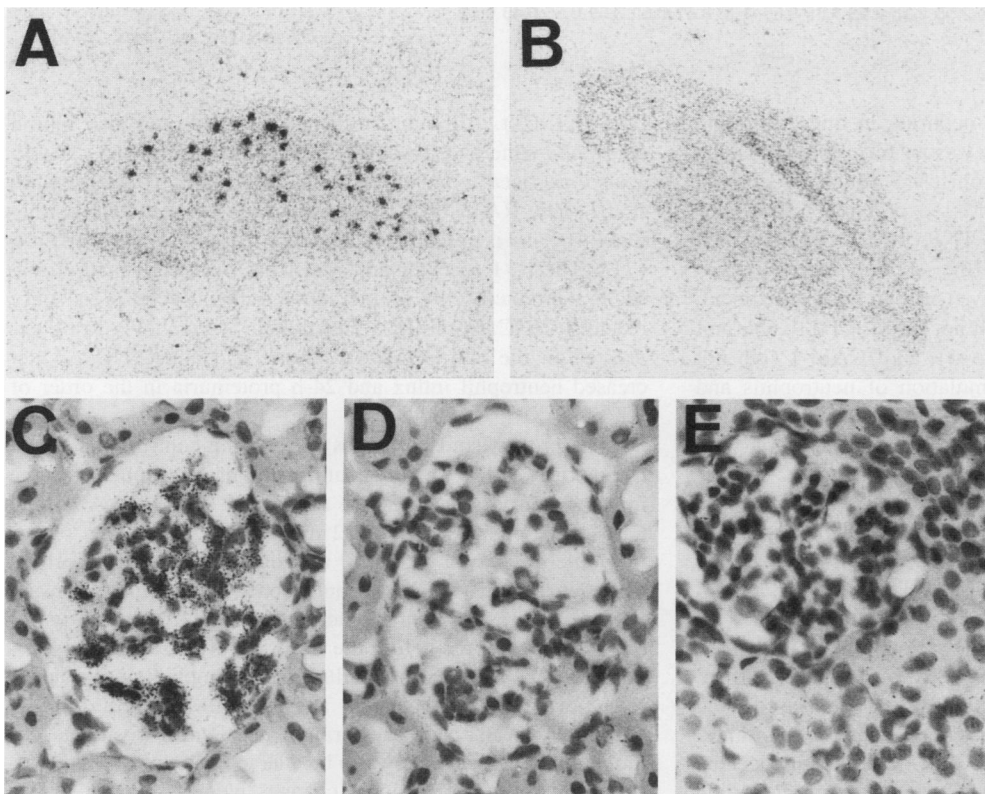

Figure 7. The in situ hybridization results are illustrated. In A, an autoradiograph of a section of kidney from a rat receiving anti-GBM Ab 4 h earlier is seen reacted with antisense MIP-2 riboprobe for comparison with the control sense MIP-2 riboprobe shown in B. The slides were exposed to x-ray film for 3 d before development. The glomeruli of the kidney in A are strongly positive. In C, the reaction of antisense and in D sense MIP-2 riboprobes are shown, respectively, from the stained section used in A. In E, the lack of reaction of the antisense MIP-2 riboprobe on normal kidney is shown. In C, MIP-2 mRNA is detected as black grains in individual glomerular cells which appear to be mesangial and intraluminal.
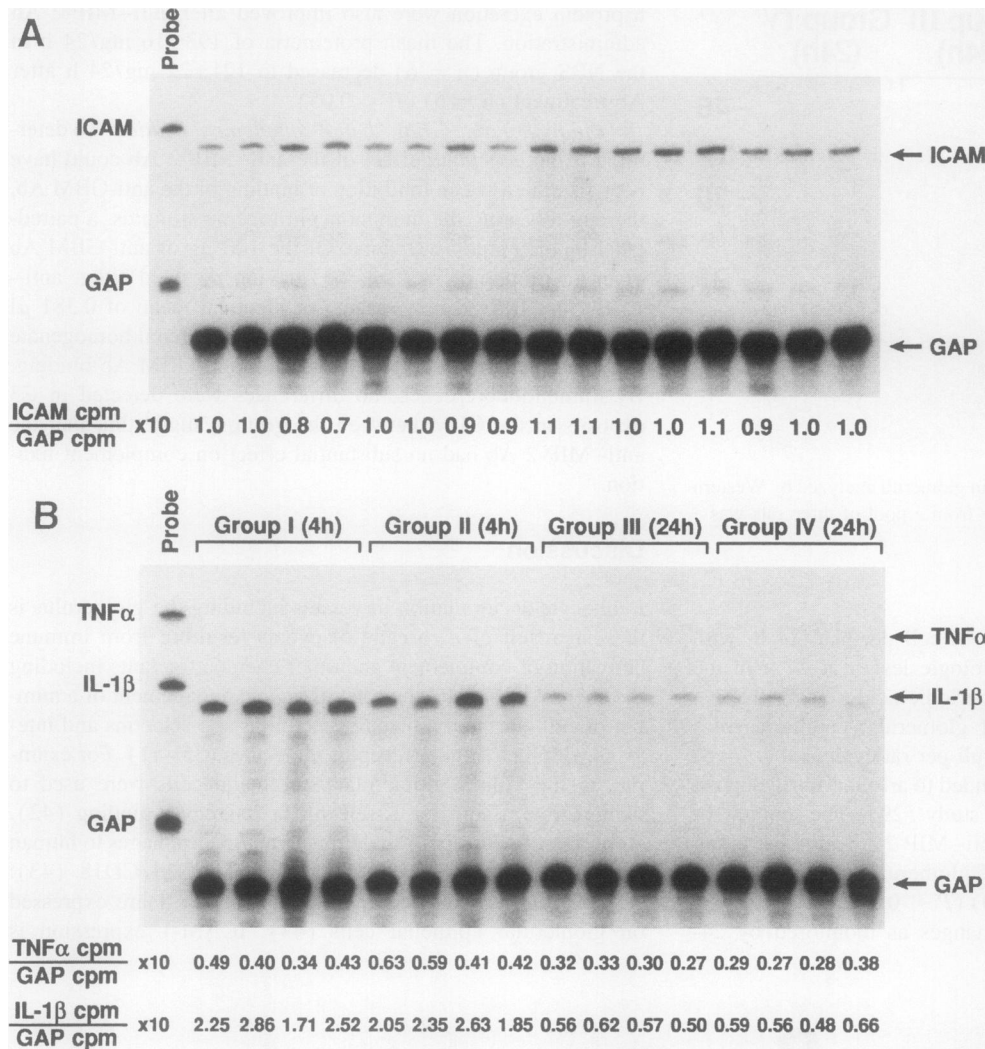
A

Probe

ICAM   ← ICAM

GAP   ← GAP

ICAM cpm / GAP cpm	x10	1.0	1.0	0.8	0.7	1.0	1.0	0.9	0.9	1.1	1.1	1.0	1.0	1.1	0.9	1.0	1.0

B

Probe

| Group I (4h) | | | | Group II (4h) | | | | Group III (24h) | | | | Group IV (24h) | | | |

TNFα   ← TNFα

IL-1β   ← IL-1β

GAP   ← GAP

TNFα cpm / GAP cpm	x10	0.49	0.40	0.34	0.43	0.63	0.59	0.41	0.42	0.32	0.33	0.30	0.27	0.29	0.27	0.28	0.38
IL-1β cpm / GAP cpm	x10	2.25	2.86	1.71	2.52	2.05	2.35	2.63	1.85	0.56	0.62	0.57	0.50	0.59	0.56	0.48	0.66

Figure 8. A shows the RNase protection results for ICAM expression in four rats from each of the four groups. No differences are apparent in mRNA expression in comparing the different time points. In *B*, the RNase protection results for TNFα and IL-1β mRNA expression are shown. The very faint TNFα bands were sufficient for AMBIS quantitation, and these as well as the IL-1β bands showed no difference relative to GAPDH (*GAP*) at the 4- and 24-h time points. Groups I and III received NRS; groups II and IV received anti–MIP-2 Ab.

upregulated in autoimmune lupus nephritis in mice (45) and has been reported to be increased in various forms of human GN (46–49). The IL-1 receptor antagonist was found to decrease neutrophil accumulation and ICAM-1 expression in the anti-GBM Ab GN model (30). Abs to TNFα or CD18 diminished neutrophil accumulation in anti-GBM Ab GN, whereas anti–E-selectin or CD11b were ineffective (50). In this same model, it has been reported that TNFα (but not IL-1), CD11b (but not CD11a), very late antigen-4 (VLA-4), and ICAM-1 (but not E-selectin) are required for accumulation of neutrophils and proteinuria (51). Abs reactive with ICAM-1 or lymphocyte function-associated antigen-1α (LFA-1α) reduced injury in anti-GBM Ab GN in the WKY rat (52, 53).

Adhesion involves not only integrin and selectin interactions with their ligands, but also activation signals provided by chemoattractants including the chemokine family (54). For example, C5a- and leukotriene B₄ (LTB₄)-induced adhesion of monocytes to human mesangial cells could be partially inhibited by Abs reactive with the common β CD18 subunit of CD11/CD18 (55); of interest, anti-selectin Abs (L, E, or P) had no effect. Members of the chemokine superfamily are being associated with glomerular injury (56). IL-8 has been identified in glomeruli from patients with IgA GN (57). IL-8 can be induced in mesangial cells and renal epithelial cells by cytokines including

IL-1 (58–62). Although a chemokine, IL-8 can also inhibit neutrophil adherence through receptor modulation (63). MCP-1 also has been found in mesangial cells (61, 64–68), as has IP-10 (69). RANTES has been found in renal tubular epithelium (70) and is upregulated in mouse mesangial cells by TNFα (71). The IL-1 receptor antagonist has been shown to inhibit MCP-1 production by glomerular mesangial cells (72). Very recently CINC/KC mRNA and protein were shown to be expressed in the anti-GBM Ab model, and anti-CINC Ab decreased neutrophil influx and 24-h proteinuria in the order of 50% (73).

The present study provides data on the contribution of the chemokine, MIP-2, in the accumulation of neutrophils of acute immune injury induced by the fixation of anti-GBM Ab to the GBM. The presence of MIP-2 mRNA expression was shown in glomerular RNA within 30 min of anti-GBM Ab injection. This elevation in MIP-2 was confirmed at the protein level using Western blot analysis of glomerular lysates. Administration of anti–MIP-2 Ab just before the injection of anti-GBM Ab produced a 40% decrease in infiltrating neutrophils at 4 h and a reduction in glomerular injury at 24 h, reflected by a decrease in intraglomerular capillary periodic acid-Schiff–positive accumulations previously shown to be characteristic of fibrin deposits (29). These morphologic changes were associated with a
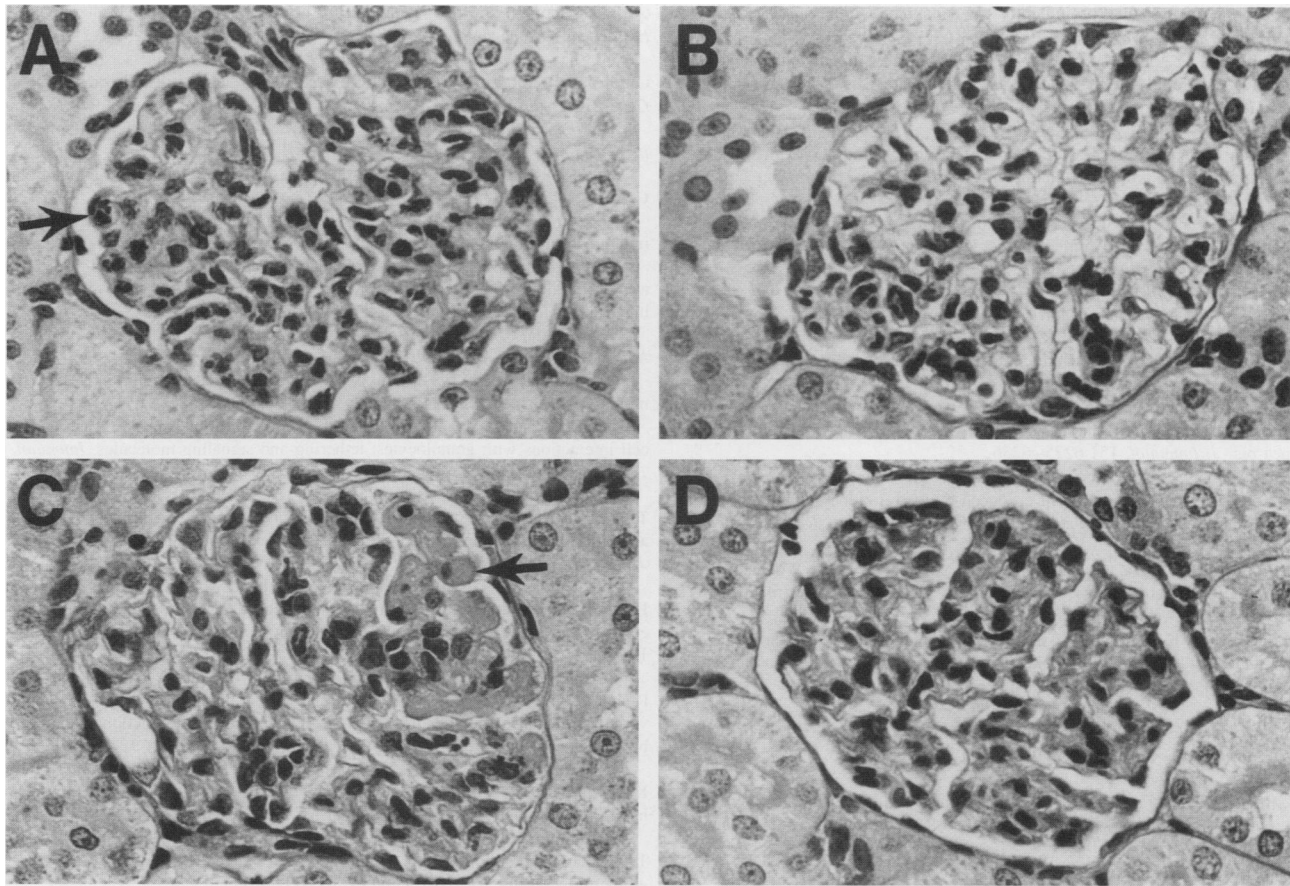
Figure 9. (A) The influx of polymorphonuclear leukocytes (*arrow*) in the NRS/anti-GBM Ab group (group I) was reduced in the anti–MIP-2/ anti-GBM Ab group in *B* (group II). In *C* (group III), periodic acid-Schiff–positive material corresponding to fibrin-containing deposits (*arrow*) were also reduced after anti–MIP-2 Ab treatment shown in *D* (group IV). Periodic acid-Schiff stain; original magnification of 400.

reduction in urinary protein excretion, confirming the beneficial effect. The effects were related to the action of the anti–MIP-2 Ab on the influx of neutrophils and not to an alteration in the underlying inducing stimulus, since no alterations in binding of anti-GBM Ab binding (paired-label isotope study), complement fixation, or IL-10, TNFα, or ICAM-1 expression could be attributed to the anti–MIP-2 Ab administration.

The anti–MIP-2 Ab-associated decrease in MIP-2 mRNA in comparison with the unchanged levels of IL-1, TNFα, and ICAM-1 mRNA suggests that the anti–MIP-2 Ab did not activate other mediator mechanisms. The decrease in MIP-2 probably related to the decrease in neutrophils. This is supported by the demonstration of MIP-2 mRNA in neutrophils by RNase protection assay (data not shown). Another interesting possibility is that the MIP-2 may induce its own gene expression, like MGSA/*gro* (74), and the anti–MIP-2 Ab may block the MIP-2 induction amplified by autocrine regulation. Additional studies are needed to confirm these observations. The anti–MIP-2 Ab effect on neutrophil influx was incomplete and C5a, LTB$_4$, PAF, and CINC/KC could contribute to a portion of the residual chemotactic activity involved in neutrophil influx. The existence of additional chemotactic peptides cannot be excluded. For example, in ongoing studies, mRNA expression of another chemotactic protein, CP-10, highly homologous with the Ca^{2+}-binding S100 protein (75, 76) has been identified in the same model (in preparation), which may contribute to some neutrophil in-

filtration after blocking MIP-2. Further study is needed to examine the role of CP-10 in this model.

Acknowledgments

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