Renal Expression of Monocyte Chemoattractant Protein-1 in Lupus Autoimmune Mice

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Abstract. Mononuclear cell infiltration in glomeruli and renal interstitium is a prominent feature of some types of glomerulonephritis, including lupus nephritis. The mechanism(s) underlying monocyte influx into the kidney is not fully understood. Recently, monocyte chemoattractant protein-1 (MCP-1) has been identified as a chemotactic factor involved in the recruitment of monocytes/macrophages in the glomeruli of rats with mesangio proliferative as well as anti-glomerular basement membrane glomerulonephritis. In the study presented here, renal MCP-1 mRNA expression in New Zealand Black x New Zealand White (NZB/W) F1 mice, a model of genetically determined immune complex disease that mimics systemic lupus erythematosus (SLE), was investigated. Northern blot analysis revealed a single 0.7 kb MCP-1 transcript of very low intensity in kidneys of 2-month-old NZB/W mice that had not yet developed proteinuria nor renal damage. Message levels, which increased markedly with the progression of nephritis and in association with mononuclear cell infiltration, were 10- and 15-fold higher in 8-10-month-old mice than in 2-month-old mice. By in situ hybridization, increased expression of MCP-1 mRNA was demonstrated in glomeruli and, even more striking, in tubular epithelial cells. Western blot analysis demonstrated increased expression of MCP-1 protein in kidneys of 10-month-old NZB/W mice, consistent with MCP-1 mRNA data. When NZB/W mice were treated with cyclophosphamide up to 12 months of age, expression of MCP-1 in the renal tissue remained low, the influx of inflammatory cells did not appear, and glomerular and tubular structures remained well preserved. These data suggest that elevated MCP-1 might act as a signal for inflammatory cells to infiltrate the kidney in lupus nephritis. (J Am Soc Nephrol 8: 720-729, 1997)

The New Zealand Black x New Zealand White (NZB/W) F1 hybrid mice spontaneously develop an autoimmune disease that closely resembles systemic lupus erythematosus in humans (1,2). The disease manifests with circulating antibodies to nucleic acid and endogenous antigens, renal immune deposits, and proteinuria. The resulting progressive glomerulonephritis is the primary cause of death in these animals. Mononuclear inflammatory cells infiltrate glomeruli and renal interstitium of mice with experimental lupus and humans with lupus nephritis (3,4). The precise mechanism(s) governing inflammatory cell recruitment into the kidney is unknown, despite sparse data implying a role of recently discovered chemoattractants and/or adhesion molecules (5,6). Among novel chemoattractants, a family of proinflammatory cytokines—chemokines (7)—including the monocyte chemoattractant protein-1 (MCP-1), a potent chemoattractant for monocytes (8,9) and for T lymphocytes (10,11), can theoretically play a pivotal role (12). MCP-1 expression and production can be regulated by cytokines and other inflammatory mediators in several cell types, including endothelial cells (13-15), mesangial cells (16,17), tubular epithelial cells (18), and monocytes/macrophages (19). Of relevance is the observation that in cultured mouse mesangial cells, binding of IgG aggregates and IgG complexes to specific Fc receptors increased MCP-1 mRNA and promoted the synthesis of the corresponding protein product (20,21). The above data can be taken to indicate that MCP-1 may act in glomerular immune complex disease as a critical molecule to the full expression of local inflammatory reaction. Actually, some data indicate that MCP-1 is overexpressed in glomeruli taken from rats with anti-thymocyte antibody-induced glomerulonephritis (22), as well as from rats with anti-glomerular basement membrane (GBM) glomerulonephritis (23,24). Moreover, immunohistochemical studies have shown increased staining for MCP-1 in renal tissue from patients with renal diseases associated with inflammatory cell accumulation in glomeruli or interstitium (23,25). Our group has recently shown that patients with active lupus nephritis had high levels of urinary MCP-1 as compared with lupus patients studied in the inactive phase of the disease or with healthy controls (26), which would suggest that in lupus nephritis,
excessive renal MCP-1 could contribute to mononuclear cell migration into the kidney.

In the study presented here, we sought to investigate in lupus-prone NZB/W mice (1) renal MCP-1 mRNA expression at different time courses during the development of renal lesions; (2) the sites of MCP-1 mRNA in the kidney; (3) the effect of immunosuppressant therapy on renal MCP-1 expression.

Materials and Methods

Experimental Design
NZB/W F1 female mice (Charles River Italia, Calco, Italy), 2 months of age at the start of the experiment, were used in these studies. Animal care and treatment were conducted in conformity with the institutional guidelines in compliance with national and international laws and policies (EEC Council Directive 86/609, OJL 358, Dec 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985). All animals were housed in a constant-temperature room with a 12-h dark, 12-h light cycle and fed a standard diet. Animals were divided into four groups, which were euthanized at 2, 6, 8, and 10 months of age, respectively. At the beginning of the study, groups 1, 2, and 3 were of \( N = 9 \) mice each, and group 4 was of \( N = 12 \) mice. At 2 months of age, before the onset of renal disease, all mice were housed in metabolic cages and 24-h urines were collected for determination of basal urinary protein excretion levels. Baseline values of urinary protein excretion ranged from 0.50 to 2.90 mg/day. Thus levels exceeding 3 mg/day were considered abnormal. Urinary protein excretion was then measured every month. Serum BUN levels were measured at the end of the experimental period. At the time that the animals were euthanized, renal tissue specimens were removed for (1) histologic analysis by light (all mice) and electron microscopy (\( N = 5 \) mice for each group, randomly selected); (2) RNA extraction and Northern blot analysis (all mice); (3) in situ hybridization (\( N = 3 \) mice for each group, randomly selected); and (4) Western blot analysis (\( N = 3 \) mice of groups 1 and 4, randomly selected).

Two additional groups of NZB/W F1 mice (\( N = 15 \) for each group) were treated with cyclophosphamide at the dose of 25 mg/kg ip once a week or vehicle—phosphate buffer solution (PBS)—starting at 3 months of age until 12 months. In all mice, urinary protein excretion was evaluated every month. At the time that the animals were euthanized, renal tissue specimens were removed for renal morphology (all mice), Northern blot analysis (all remaining mice of vehicle group and \( N = 6 \) mice, randomly selected, for cyclophosphamide group), and in situ hybridization. For comparison, five CD-1 (ICR) BR untreated mice were followed until 12 months of age.

Renal Morphology

Light Microscopy. Fragments of renal cortex were fixed in Dubosq-Brazil, dehydrated in alcohol, and embedded in paraffin. Sections (3 \( \mu m \) each) were stained with hematoxylin and eosin, Masson’s trichrome, and periodic acid-Schiff’s reagent. Each biopsy included at least 100 glomeruli. Glomerular endocapillary hypercellularity was quantitated by a scoring system from 0 to 3+ (0, no hypercellularity; 1+, mild; 2+, moderate; and 3+, severe). Extracapillary proliferation was graded from 0 to 3+ (0, no hypercellularity; 1+, <25% of glomeruli involved; 2+, 25%–50% of glomeruli involved; and 3+, >50% of glomeruli involved). Glomerular deposits were graded from 0 to 3+ (0, no deposits; 1+, <25% of glomeruli involved; 2+, 25%–50% of glomeruli involved; and 3+, >50% of glomeruli involved). Glomerular sclerosis was graded from 0 to 3+ (0, no sclerosis; 1+, sclerosis affecting <25% of glomeruli; 2+, sclerosis affecting 25%–50% of glomeruli; and 3+, sclerosis affecting >50% of glomeruli). Tubular (atrophy, casts, and dilatation) and interstitial changes (fibrosis and inflammation) were graded from 0 to 3+ (0, no changes; 1+, changes affecting <25% of the sample; 2+, changes affecting 25%–50% of the sample; and 3+, changes affecting >50% of the sample). At least 100 glomeruli were scored per mouse, and 10–18 fields per mouse were examined at low magnification (10×) for histologic scoring of the interstitium. All renal biopsies have been analyzed by the same pathologist, blind to the nature of the experimental groups.

Electron Microscopy. Small fragments of kidney were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 4 h at 4°C. Samples were washed in cacodylate buffer and subsequently postfixed in 1% osmium tetroxide for 1 h. After a brief wash in cacodylate buffer, they were dehydrated through ascending grades of alcohol and embedded in Epon resin. Sections were cut on an LKB V ultramicrotome (Bromma, Sweden). Semithin sections were stained with toluidine blue in borax and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and then they were examined with a Zeiss EM 109.

RNA Isolation and Northern Blot Analysis
Total RNA was isolated from mouse kidneys by the guanidinium isothiocyanate/cesium chloride procedure, as previously described (27). For mRNA preparation, total RNA of kidneys of each group of NZB/W mice was pulled together. Poly (A)+ RNA was selected by oligo (dT)-cellulose column chromatography (mRNA separator, Clontech, Palo Alto, CA). Then, 7 \( \mu g \) of mRNA were fractionated on 1.2% agarose gel and blotted onto synthetic membranes (Gene Screen Plus, New England Nuclear, Boston, MA). Plasmid containing murine JE/MCP-1 probe was kindly provided by Dr. Charles D. Stiles (Harvard Medical School and Dana-Farber Cancer Institute, Boston, MA). MCP-1 mRNA was detected by using the 577 basepair (bp) of MCP-1 cDNA (28). The cDNA fragment of MCP-1 was labeled with \( \alpha^{32}PdCTP \) by random-primed method (29). Membranes were hybridized for 20 h at 60°C with 1.5 \( \times 10^6 \) cpm-labeled probe, and the filters were washed as previously described (30) and exposed to x-ray film for autoradiography. Membranes were subsequently probed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (31), taken as the internal standard of equal loading of the samples on the membrane. MCP-1 mRNA optical density was normalized to that of the constitutively released GAPDH gene expression. Data of MCP-1 mRNA levels (\( N = 3 \) experiments) were analyzed by one-way analysis of variance using the Tukey test for multiple comparisons. Statistical significance was defined as \( P < 0.05 \).

Preparation of Digoxigenin-Labeled Murine JE/MCP-1 Riboprobes
The murine JE/MCP-1 antisense and sense RNA probes were synthesized and labeled by in vitro transcription using digoxigenin-labeled uridine triphosphate. A 577-bp murine JE/MCP-1 cDNA was cloned into the EcoRI site of the pGEM-1 vector between SP6 and T7 promoters (gift from Dr. Charles D. Stiles) The plasmid was linearized with the appropriate restriction enzymes, Sacl (antisense murine JE/MCP-1, T7 polymerase) and SpeI (sense murine JE/MCP-1, SP6 polymerase), and purified by phenol/chloroform extraction and ethanol precipitation. Linearized plasmid was resuspended in diethyl pyrocarbonate (DEPC)-treated water. Transcription of 1 \( \mu g \) linearized plasmid was performed using a DIG RNA labeling Kit (Boehringer-
Mannheim Biochemica, Mannheim, Germany). The reaction products were ethanol precipitated and stored at 70°C until use. Labeling efficiency of the riboprobe was estimated by comparison with 10-fold serial dilutions of a digoxigenin-labeled control riboprobe (Boehringer Mannheim Biochemica) and direct detection of the labeled riboprobe with anti-digoxigenin antibodies. Riboprobe concentrations were adjusted to be equivalent on the basis of the labeling efficiency before use in the in situ hybridization studies.

**In Situ Hybridization**

Ten percent neutral buffered formalin-fixed and paraffin-embedded renal tissues were cut at 4 μm and floated onto 2% 3-aminopropyltriethoxysilane (APES) (Sigma Chemical, St. Louis, MO) coated slides. Sections were heat-fixed for 30 min at 65°C and deparaffinized. After a 30-min incubation in 5 mmol/L levamisole, the sections were washed in PBS and in DEPC-treated water for 5 min, respectively, and then immersed in 0.2 mol/L HCl for 20 min. Then, the sections were deproteinized by digestion with 40 μg/mL proteinase K (Sigma) for 10 min at 37°C, washed in 0.2% glycine for 5 min twice and in PBS for 10 min, and postfixed with 1.5% paraformaldehyde-1.5% glutaraldehyde for 1 min. After rinsing twice with PBS in PBS, sections were dehydrated through a graded ethanol series and air dried. The sections were hybridized with the RNA probes at the final concentrations of 0.1-0.5 ng/μl in 2 × SSC. 10% dextran sulfate, 1 × Denhardt’s solution, 20 mM Vanadyl Ribonucleoside Complex (GIBCO BRL, Life Technologies, Gaithersburg, MD), 0.1 M sodium phosphate under sealed coverslips and incubated overnight in a moist chamber at 42°C. They were washed in 0.2 × SSC and then blocked with a buffer blocking solution (50 mg/mL skimmed dried milk, 150 mM NaCl in 100 mM Tris HCL, pH 7.8) at room temperature for 15 min, and the sections were incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer-Mannheim Biochemica) at the dilution of 1:750 for 30 min at 37°C. Colorimetric detection with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate (Boehringer-Mannheim Biochemica) was then performed, and the sections were mounted in 60% glycerol and examined by light microscopy. The negative controls included: (1) hybridization with the sense probe, (2) RNase (100 μg/ml in 10 mM Tris HCL, pH 8.0, 1 mM ethylenediaminetetraacetate) pretreatment before hybridization, and (3) omission of either the antisense RNA probe or the anti-digoxigenin antibody.

**Western Blot Analysis**

For Western blot analysis, kidneys from NZB/W 2- and 10-month-old mice were homogenized in lysis buffer containing 0.1 M Tris pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, and 1 mM Pefabloc (Boehringer-Mannheim Biochemica). The samples were incubated for 20 min at room temperature with 0.5 mg/ml DNase I (Boehringer-Mannheim Biochemica) and 10 mM MgCl₂, and then centrifuged at 15,000 rpm for 2 h. After determination of protein concentration in the supernatant by the Coomassie method (Bio-Rad, Hercules, CA), samples (50 μg/lane) were electrophoresed on 15% SDS-polyacrylamide gels in reducing conditions. Proteins were then transferred to a nitrocellulose filter for 2 h at 100 V. To block nonspecific binding sites, the membrane was incubated in PBS + 5% low-fat dry milk for 1 h at room temperature, and then for 2 h at room temperature with purified monoclonal antibodies: hamster anti-mouse MCP-1 2H5 (0.5 μg/ml) (32) or rat anti-mouse MCP-1 ECE.2 (0.8 μg/ml), prepared as described below. After incubation with peroxidase conjugated rabbit anti-hamster IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA) for 2H5 and peroxidase conjugated sheep anti-rat IgG (Amersham Life Science, Amersham, UK) for ECE.2 for 1 h at room temperature, the reactivity was detected with ECL detection reagent (Amersham Life Science). Molecular weights of the immunoreactive bands were estimated by running aside a mixture of molecular standards (GIBCO, Grand Island, NY). Recombinant mouse MCP-1 was used as reference (32). To confirm the specificity of the reaction, in one experiment one of two identical membranes was incubated with ECE.2 and the other one with ECE.2 in the presence of the MCP-1 peptide it recognizes.

**Monoclonal Antibody Generation**

Male Lewis rats (Charles River Italia, Calco, Italy) were immunized with a synthetic peptide—prepared as previously described (33)—spanning residues 102-130 of mouse MCP-1 (32) conjugated with keyhole limpet hemocyanin (Sigma Chemical, St. Louis, MO). Hybridomas were generated as previously reported (34) and screened for MCP-1 reactivity with enzyme-linked immunosorbent assay. Monoclonal antibody ECE.2 (IgG1 class) was specific for mouse, but not human, recombinant MCP-1 and was purified from serum-free supernatants by ammonium sulfate precipitation and size-exclusion chromatography (35).

**Analytical**

Urinary protein concentration was determined by the Coomassie blue G dye-binding assay with bovine serum albumin as standard (36). Renal function was assessed as BUN on serum samples using an enzymatic UV Rate by Sincron CX-5 (Beckman, Fullerton, CA). BUN levels exceeding 30 mg/dl were considered abnormal (normal range in our laboratory: 14-29 mg/dl). BUN data were expressed as mean ± SE.

**Results**

**Life Survival in NZB/W Mice**

Up to 6 months of age, all NZB/W mice were alive. At 8 and 10 months of age, the percentage of survival was 67% and 42%, respectively.

**Time Course of Proteinuria and Renal Function in NZB/W Mice**

Cumulative frequency of proteinuria >3 mg/day in NZB/W mice at different stages of the disease is shown in Table 1. At 2 months of age, none of the animals was proteinuric. Therefore, cumulative percentage of proteinuric mice progressively increased over time, reaching 92% at 10 months of age.

Renal function—as evaluated by serum BUN measurements—was normal up to 6 months of age (2 months, 24.4 ± 2.1; 6 months, 21.9 ± 1.3 mg/dl), but deteriorated at 8 and 10 months. This was reflected in elevations of BUN values (8 months, 139.5 ± 45.6; 10 months, 133.7 ± 39.8 mg/dl).

**Renal Morphologic Changes in NZB/W Mice at Different Stages of the Disease**

Data of renal morphologic analysis by light microscopy in NZB/W mice at different months of age are shown in Table 1. At 2 months, mice did not exhibit changes in the glomeruli, interstitium, tubules, and vessels. At 6 months, light microscopy analysis revealed only mild glomerular changes characterized by mild endocapillary hypercellularity not associated
with obvious damage in the tubules, interstitium, and vessels. At 8 and 10 months, glomerular changes were pronounced, with endocapillary hypercellularity associated with a focal extracapillary proliferation. Immune type of deposits was detected in the mesangium and on subendothelial aspect of GBM. Interstitial inflammation, fibrosis, and tubular damage were severe. Global or segmental sclerotic changes were confined to <25% of glomeruli.

By electron microscopy analysis of kidneys from NZB/W mice at 2 months of age, the only glomerular lesion consisted of focal electron-dense deposits within the GBM or on the subepithelial aspect of GBM in some animals. At 6 months, glomerular changes included inflammatory cells in the capillary lumina, as well as diffuse electron-dense deposits in the mesangium and on the subepithelial aspect of GBM. At 8 and 10 months, lesions worsened, with more inflammatory cells accumulating in the glomerular capillaries, and numerous mesangial, subendothelial, intramembranous, and subepithelial electron-dense deposits. These findings agree with previously published observations in this model (37, 38).

Renal MCP-1 Gene Expression in NZB/W Mice

The time course of renal MCP-1 gene induction—monitored by Northern blot analysis—during the evolution of the disease in NZB/W lupus mice is given in Figure 1. A single 0.7 kb MCP-1 mRNA transcript of very low intensity was detected in kidneys from 2-month-old NZB/W mice. Message levels increased markedly with time. Thus, as revealed by densitometric analysis of the autoradiographic signals, renal MCP-1 mRNA levels in 6-, 8-, and 10-month-old mice were 2- (P < 0.05), 10-, and 15- (P < 0.01) fold higher, respectively, than those of 2-month-old mice (Figure 2).

### Table 1. Cumulative frequency of proteinuria (>3 mg/day) and renal histology in NZB/W mice at different stages of the disease

<table>
<thead>
<tr>
<th>Months of Age</th>
<th>Cumulative % Proteinuric Mice*</th>
<th>Endocapillary Hypercellularity</th>
<th>Extracapillary Proliferation</th>
<th>Glomerular Hyaline Deposits</th>
<th>Glomerular Sclerosis</th>
<th>Interstitial Fibrosis or Inflammation</th>
<th>Tubular Damage</th>
</tr>
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<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>6</td>
<td>0</td>
<td>0.37 (0–1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.11 (0–1)</td>
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<tr>
<td>8</td>
<td>55</td>
<td>1.88 (1–2)</td>
<td>0.44 (0–1)</td>
<td>1.33 (0–3)</td>
<td>0.44 (0–1)</td>
<td>1.44 (0–3)</td>
<td>1.22 (0–3)</td>
</tr>
<tr>
<td>10</td>
<td>92</td>
<td>1.72 (1–2)</td>
<td>1.00 (0–3)</td>
<td>2.09 (1–3)</td>
<td>0.91 (0–3)</td>
<td>1.81 (1–3)</td>
<td>1.72 (0–3)</td>
</tr>
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* Each point reflects the current level of proteinuria in surviving mice, as well as the last measurement in deceased mice. Values of renal histological parameters are mean scores; ranges are in parenthesis.

Figure 1. Time-dependent renal expression of MCP-1 mRNA in NZB/W lupus mice from a representative Northern blot (N = 3 experiments). mRNA (7 μg) obtained from pooled kidneys of NZB/W mice at 2 (N = 9), 6 (N = 9), 8 (N = 6), and 10 (N = 5) months of age was blotted onto synthetic membranes which were hybridized sequentially with α-32P-labeled murine JE/MCP-1 (top) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (bottom) cDNA probes.

Figure 2. Corresponding densitometry of the autoradiograph reported in Figure 1 shows MCP-1 mRNA expression in kidneys from NZB/W mice at different stages of the disease. The optical density of the autoradiographic signals was quantitated and calculated as the ratio of MCP-1 to GAPDH mRNA. The mRNA levels of 6, 8, and 10 months were calculated by assuming the optical density of 2 months as unit.
Figure 3. Photomicrographs showing MCP-1 mRNA in kidneys from NZB/W mice and a control CD mouse by in situ hybridization (digoxigenin d-UTP-labeled murine MCP-1 probe, alkaline phosphatase reporter system). Hybridization signal can be seen in some glomerular and tubular epithelial cells in a kidney from a 2-month-old NZB/W mouse (a). The signal is more intense both in glomeruli and tubules in
In Situ Hybridization

In situ hybridization was performed with antisense JE/MCP-1 RNA probe to localize MCP-1 mRNA expression at the cellular level on kidneys of NZB/W lupus mice in different stages of the disease. Hybridization signal of mainly perinuclear or cytoplasmic pattern was clearly detectable in the renal cortex. In 2-month-old mice, the signal was observed in glomerular cells. When these cells were situated peripherally in glomerular tuft, they could be confidently identified as visceral and parietal epithelial cells. Some endothelial and mesangial cells were also labeled. MCP-1 mRNA was also present in some tubular epithelial cells (Figure 3a). The intensity of the labeling increased progressively in NZB/W mice at 6, 8, and 10 months of age, in agreement with the results of the Northern blot analysis (Figure 3b-f). The most intense signals were found in tubules. At 6 months, increased MCP-1 mRNA staining in glomeruli was associated with endocapillary proliferative lesions. The staining was present both in resident glomerular cells and in infiltrating cells. The tubular MCP-1 mRNA staining at this time was not yet clearly associated with the presence of inflammatory cells in interstitium (Figure 3b). Conversely, there were interstitial infiltrates in kidneys of 8- and 10-month-old mice, and the inflammatory cells stained positive for MCP-1 mRNA. Most of the staining, however, was found in the tubules, which were also adjacent to sites of accumulation of inflammatory cells (Figure 3c-f). Signal in areas of glomerular sclerosis was weak or absent. No hybridization was detected in negative control sections pretreated with RNase before incubation with antisense MCP-1 RNA probe, or in sections incubated with the corresponding sense MCP-1 probe (Figure 3g).

Renal MCP-1 Protein Expression in NZB/W Mice

As shown in Figure 4, Western blot analysis of proteins extracted from kidneys of 10-month-old NZB/W mice, performed with two monoclonal antibodies against mouse MCP-1 revealed three bands with an apparent molecular mass of approximately 30, 18, and 14 kd corresponding to heavily and partially glycosylated forms of MCP-1 (32,39). By contrast, in the kidney extract of 2-month-old NZB/W mice, only a faint band of 14 kd was detected, and no higher molecular weight bands were observed, thus confirming our results at the mRNA level. The specificity of the reaction was confirmed by the fact that no band was observed when Western blotting was performed on 10-month-old NZB/W kidney extract with ECE.2 in the presence of the peptide it recognizes (data not shown).

Effect of Cyclophosphamide on Renal MCP-1 Expression in NZB/W Mice

Two groups of NZB/W mice up to 12 months of age were treated with cyclophosphamide or vehicle. An additional group of 12-month-old CD1(ICR)BR normal mice was used as control. At the end of the observation period, all cyclophosphamide-treated mice were alive, and none of them had proteinuria of >3 mg/day. Only mild glomerular changes were observed by light microscopy analysis (mean scores, endocapillary hypercellularity: 0.6; extracapillary hypercellularity: 0; hyaline deposits: 0.3; tubulo-interstitial damage: 0; glomerular sclerosis: 0). By contrast, in the vehicle-treated group, only 20% of mice survived, and the cumulative percentage of proteinuric mice was 93%. Glomerular and tubulo-interstitial changes were severe (mean scores, endocapillary and extracapillary proliferation: 1.3; hyaline deposits: 2; glomerular sclerosis: 1.7; tubulo-interstitial damage: 1.7). Northern blot analysis showed that cyclophosphamide treatment prevented the increase in renal MCP-1 mRNA that occurred in untreated NZB/W lupus mice of the same age (Figures 5 and 6, P < 0.01). Renal MCP-1 transcript levels of 12-month-old control mice that did not exhibit any glomerular or tubulo-interstitial damage (mean score for all the parameters considered: 0) remained lower than levels of untreated 12-month-old lupus mice (P < 0.01), thus excluding that upregulation of MCP-1 in NZB/W lupus mice was a consequence of aging. In accordance with Northern blot experiments, in situ hybridization showed that in kidneys of cyclophosphamide-treated mice (Figure 3j), the intensity of MCP-1 mRNA staining was dramatically decreased compared with that of untreated mice of the same age (Figure 3i) and similar to that observed in 2-month-old NZB/W mice (Figure 3a) that had not yet developed the disease. In kidneys of 12-month-old normal mice, the signal for MCP-1 was comparable to that observed in 2-month-old NZB/W mice (Figure 3h).

Discussion

The first finding of the study presented here is that MCP-1 mRNA is upregulated in the kidneys of mice with lupus nephritis. Specifically, using Northern blot analysis, we have demonstrated that renal MCP-1 message levels increased progressively with the development of the disease in NZB/W lupus mice, being 10- and 15- fold higher at 8 and 10 months than at 2 months of age, when mice showed no sign of renal disease. Overexpression of MCP-1 in renal tissue paralleled mononuclear cell accumulation, as indicated by the morphologic analysis performed at different time points during the development of the renal disease.
Infiltrates of mononuclear cells within the glomeruli in lupus, as in many other glomerulonephritis conditions, precede the development of glomerular structural lesions and are very likely to contribute to tubulo-interstitial damage (40, 41). Old studies established the fundamental role of mononuclear cells in initiating and monitoring renal injury in immune complex disease. Thus, the evidence is available to show that macrophage depletion abrogated proteinuria and effectively prevented subsequent injury in experimental glomerulonephritis (42). More recently, the myriad of macrophage proinflammatory functions are being elucidated, and the various molecules responsible for the different effects are now at least partially identified (43, 44). Among these are cytokines synthesized and released by macrophages infiltrating the glomerulus, either in human or experimental immune-mediated glomerulonephritis (43, 44), platelet-activating factor, and reactive oxygen species (45). Other data tend to suggest that infiltrating macrophages are involved in renal disease progression to the extent that they participate in glomerular crescent formation and appear directly responsible for glomerular fibrin deposition by virtue of their surface-related property of expressing procoagulant activity (46, 47). Despite so many studies on the role of monocytes/macrophages in glomerulonephritis, data on signaling events to drive them into the glomerulus are very few. Advances in basic mechanisms governing macrophage chemotaxis and on the different proteins involved have provided an opportunity to unravel the issue. In this context, we have concentrated on MCP-1, a member of the βchemokine (C-C chemokine) subfamily (7, 48), which corresponds to the product of the early response gene, JE, of the mouse (19). MCP-1 is a chemotactic factor (8–11) that, in addition, induces the respiratory burst in human monocytes (49); activates monocytes to synthesize cytokines, including interleukin-1 and interleukin-6 (50); and upregulates adhesion molecule expression on monocytes (50), which results in increased adhesion of monocytes to endothelial cells (51). All of these properties make MCP-1 a potent pro-inflammatory molecule. Experimental data are available showing that MCP-1 can be regarded as a good candidate in the sequence of signaling events leading to kidney infiltration by inflammatory cells. In rat anti-Thy 1.1 glomerulonephritis, expression of glomerular MCP-1 increased markedly in the early phase of mesangial immune complex formation 30 min after disease induction, when infiltration of monocytes/macrophages started to occur (22). MCP-1 message levels were reduced during the subsequent phase of mesangiolysis (at 24 h) and increased again during proliferative glomerulonephritis (at 5 and 21 days). Decomplementation of rats
prevented MCP-1 induction and glomerular influx of monocytes/macrophages. Other studies showed that in rats with anti-GBM glomerulonephritis, glomerular MCP-1 mRNA increased in association with the monocytic influx and correlated with expression of immunoreactive MCP-1 in the nephritic glomeruli (23).

To study the distribution of MCP-1 mRNA in the kidney, we performed in situ hybridization experiments on renal tissue from NZB/W lupus mice of different ages. MCP-1 mRNA was detected in the glomerular epithelial, endothelial, and mesangial cells, as well as in tubules and interstitium. The intensity of the signal increased progressively with the evolution of the disease, in accordance with the Northern blot analysis results. Western blot experiments revealed an increased expression of MCP-1 protein in the renal tissue of 10-month-old as compared with 2-month-old NZB/W mice, which was consistent with MCP-1 mRNA data. Our in vivo findings agree with in vitro studies showing the capability of both glomerular and tubular epithelial cells in culture to express and produce MCP-1 in response to cytokines and other inflammatory mediators (16–18), a phenomenon that, at least in glomerular mesangial and endothelial cells, is mediated by the activation of NF-kB and AP-1 transcription factors (52,53). In mice with lupus nephritis, gene expression of interleukin-1 and tumor necrosis factor α is increased in the renal cortex (54), which might imply that cytokines act as regulatory molecules for the observed MCP-1 overexpression. Mesangial cells are also induced by IgG and immune complexes to release MCP-1 upon engagement of Fc receptors (20,21). In the study presented here, increased MCP-1 mRNA was detected in glomeruli at 6 months, a time of early accumulation of mononuclear cells in capillary lumina. Additionally, consistent with previous studies (37,38), we detected diffuse electron-dense deposits by electron microscopy in the mesangium at this time. These findings together may suggest that immune complexes, particularly those deposited in the mesangium, act to trigger MCP-1-mediated pathways of glomerular mononuclear cell accumulation in NZB/W mice, similar to other experimental diseases like anti-Thy 1.1 (22) and anti-GBM glomerulonephritis (23,24) in rats.

Our finding of a remarkable increase of MCP-1 mRNA in tubular cells of NZB/W lupus mice of 8 and 10 months of age might explain, at least in part, the accumulation of mononuclear cells observed in renal interstitium. Recent experiments by Harris et al. (55) have shown an upregulation of MCP-1 mRNA expression in proximal tubular cells in culture exposed to albumin and transferrin-iron at concentrations similar to those found in proteinuric urine. It is tempting to speculate that in lupus nephritis, tubular overexpression of MCP-1 might be the consequence of the excessive tubular reabsorption of proteins ultimately leading to interstitial inflammation.

Patients with lupus nephritis studied in the active phase of the disease had higher levels of urinary MCP-1 than in the inactive phase (26). Administration of high-dose steroids to patients with active disease significantly reduced urinary MCP-1. In harmony with the above human findings are additional data from the study presented here, indicating that cyclophosphamide given to NZB/W mice up to 12 months of age effectively limited proteinuria and renal damage and prevented upregulation of renal MCP-1 gene as assessed by Northern blot and in situ hybridization experiments.

When interpreting data on the effect of steroids and cyclophosphamide on renal MCP-1 upregulation in lupus nephritis, the possibility that these drugs inhibit B cell activation and antibody synthesis should not be overlooked. This will prevent immune complex formation, regardless of the contributing role of MCP-1.

In summary, our results show a progressive, time-dependent upregulation of renal MCP-1 gene during the course of nephritis in NZB/W lupus mice that closely parallels the time course of mononuclear cell infiltration in the kidney. These data can be taken to suggest that MCP-1 acts as a major signaling molecule for mononuclear cell recruitment at the glomerular and tubulo-interstitial level, at least in murine models of SLE. In the past few years, attempts to block cytokine activity have been made by neutralizing antibodies, receptor antagonists, or soluble receptors (56). Molecules that effectively antagonize the biological activity of MCP-1—if available in the near future—may possibly find an important role in the therapy of lupus nephritis.

Acknowledgments
We thank Daniela Cavallotti for technical assistance. We are greatly indebted to Dr. Charles D. Stiles (Department of Microbiology and Molecular Genetics, Harvard Medical School, and Dana-Farber Cancer Institute, Boston, MA) for providing JE/MCP-1 probe. We also thank Dr. Alberto Mantovani (Mario Negri Institute, Milano, Italy) for discussion and guidance in preparation of anti-MCP-1 antibody. Dr. Luca De Gioia (Mario Negri Institute, Milano, Italy) for preparing MCP-1 synthetic peptide, and Dr. Paolo Ruggiero (Centro Ricerche Dompé, Biotecnologie, L’Aquila, Italy) for helpful advice.
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