Chemokine and Chemokine Receptor Expression during Initiation and Resolution of Immune Complex Glomerulonephritis

Abstract. Chemokines participate in leukocyte infiltration, which plays a major role in glomerular injury during immune complex glomerulonephritis (IC-GN). Because target cell expression of chemokine receptors (CCR) is thought to mediate leukocyte migration, the expression pattern of chemokines and CCR in a model of IC-GN was examined. The transient course and predominant glomerular pathology of this model allows the examination of both the induction and resolution phases of IC-GN. GN was induced in mice by daily apoferritin injection for 2 wk. Urine samples and kidneys were obtained at 1, 2, and 4 wk. Albuminuria was noted at 2 wk, but resolved after 4 wk. This was associated with glomerular IC deposits and mesangial proliferation. Glomerular macrophage infiltration was prominent at 1 and 2 wk, which resolved at 4 wk. Expression of monocyte chemoattractant protein-1 (MCP-1) and RANTES mRNA was upregulated at week 1 and decreased to control levels at weeks 2 and 4. The expression was localized to glomeruli by in situ hybridization and immunohistochemistry. The mRNA of CCR1, CCR2, and CCR5 but not CCR3 or CCR4 were upregulated at week 1 and decreased at weeks 2 and 4. Expression of CCR5 was located to the glomerulus by in situ hybridization and quantitative reverse transcription-PCR of isolated glomeruli. In summary, in a model of transient IC-GN, MCP-1 and RANTES and their receptors CCR1, CCR2, and CCR5 are expressed early and are already downregulated at the peak of proteinuria and leukocyte infiltration. Resolution of glomerulonephritis is associated with a return to baseline of chemokine and CCR expression. Therefore, it is concluded that glomerular MCP-1 and RANTES production directs circulating leukocytes that express CCR1, CCR2, and CCR5 into the glomerulus. After initiating GN, MCP-1 and RANTES and their receptors are readily downregulated.
renal disease are still sparse. In human renal biopsies, CCR5 was localized to interstitial mononuclear cells, predominantly T cells, in various kidney diseases (13). In human crescentic GN, CCR2 mRNA expression was found in crescentic lesions on interstitial leukocytes (14). CXCR4 was found on infiltrating leukocytes during human renal allograft rejection (15). Furthermore, CCR1, CCR2, and CCR5 mRNA were upregulated in cortical isolates from mice with NSN with crescentic GN and a strong periglomerular leukocyte accumulation (5,16).

NSN is the most widely used experimental model of immune complex GN (IC-GN). NSN is characterized by a necrotizing and crescentic GN in mice, accompanied by a strong periglomerular and interstitial leukocytic infiltrate resembling human rapidly progressive GN (5,7,17). Therefore, murine NSN cannot be considered as a model of isolated GN. Consistent with the prominent tubulointerstitial injury, a predominantly tubular expression of MCP-1 was observed in wild-type mice with NSN (8). Concordantly, mice that were deficient of MCP-1 were protected from tubulointerstitial injury, in correlation with a decreased number of interstitial macrophages, but not from glomerular damage (8). A model of an isolated and reversible IC-GN resembling human diseases such as postinfectious GN, IgA nephropathy, cryoglobulin-related GN type II, or early lupus nephritis has not been studied.

We therefore established a murine model of reversible IC-GN by daily injection of apoferritin for 2 wk, which allowed us to study the expression of chemokines and their receptors during both the induction and resolution phases of IC-GN. Horse apoferritin-induced GN (HAF-GN) was characterized by mesangial IC deposition, diffuse mesangial proliferation, a glomerular influx of macrophages, and proteinuria without significant tubulointerstitial injury. We observed that the maximum mRNA expression of MCP-1 and RANTES and their receptors CCR1, CCR2, and CCR5 occurs early in the initiation phase of GN and before the onset of proteinuria. CCR5 was localized to glomeruli only. At the peak of proteinuria, the expression of both chemokines and CCR decreased despite persistent glomerular leukocyte infiltration. Resolution of GN with normalization of the glomerular leukocyte count and the proteinuria was associated with return to control of chemokine and CCR expression. These data point toward a role of MCP-1 and RANTES for the initiation of glomerular leukocyte influx, which then results in proteinuria.

### Induction and Evaluation of IC-GN

GN was induced by daily intraperitoneal injections of 4 mg of HAF in 80 μl of 0.1 M sodium chloride (Sigma-Aldrich Chemicals, Steinheim, Germany) for 14 consecutive days (18). Controls received injections of 80 μl of 0.1 M sodium chloride.

Body weight was determined at the beginning of the study and at weekly intervals. Blood samples were collected from each animal 1 wk before the beginning and at the end of the study by bleeding from the retro-orbital venous plexus under general anesthesia with inhaled ether. After centrifugation, all serum samples were stored at −80°C until analyzed. Spot urine samples were collected from each animal initially and at the end of the study. For the determination of urine protein/creatinine ratio (Up/Ucr [μg/ml]), serum creatinine and urea nitrogen concentrations were determined using a Hitachi 717 Autoanalyzer (Roche, Mannheim, Germany), and urine albumin concentration was measured using a mouse albumin enzyme-linked immunosorbent assay (ELISA) kit (Albuwell M, Exocell Inc., Philadelphia, PA).

The immune response to the injected antigen was assessed by measuring anti-HAF titers based on a previously described ELISA method (19). In brief, 96-well polystyrene microtiter plates (Greiner, Munich, Germany) were coated at 4°C overnight with 0.1 mg/ml HAF in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M NaH2PO4 [pH 7.4]). After washing the plates with 0.05% Tween-20 (Sigma-Aldrich, Deisenhofen, Germany) in PBS, nonspecific binding of protein to the wells was blocked by 1 h of incubation with 1% bovine serum albumin at room temperature followed by three washes with 0.05% Tween-20 in PBS. Serial dilutions of serum samples were added in duplicate for 1 h at room temperature. After three washes with 0.05% Tween-20 in PBS, a 1:2000 dilution of horseradish-peroxidase anti-mouse Ig antibody (P260; Dako, Hamburg, Germany) was added for 1 h. Peroxidase was developed with 2,2- amino-bis(3-ethylbenzothiazoline)-6-sulfonate substrate (Boehringer Mannheim, Germany), and the optical density was measured at 450 nm applying a microtiter plate reader (Molecular Devices Corp., Munich, Germany). Serum samples of HAF-immunized mice were compared with nonimmunized mice. Pooled serum from three mice that were immunized three times at 2-wk intervals by intramuscular injections with 0.05 mg of HAF in TiterMax Gold Adjuvants (Sigma-Aldrich) served as positive controls in the ELISA.

### Morphologic Evaluation

From each mouse, the left kidney was used for histologic assessment. Each kidney was divided horizontally into two halves by a midline cut. For quantitative analysis, 2-μm-thick slices cut from the horizontal cut surface were used. Every fifth of 15 subsequent slices, chosen by systematic uniformly random sampling, were stained and analyzed. Intraglomerular cells were counted in at least 30 cortical glomeruli per section, selected by uniformly random sampling, from each animal and were analyzed. Subcapsular glomeruli (up to 50 μm) were not evaluated to avoid stain-related edge artifacts. Glomeruli were assessed only when more than 10 capillary loops were present to exclude evaluation of pole cuts. Only cells within the glomerular tuft were counted. Cells were considered periglomerular when localized adjacent to the outside of Bowman’s capsule. Interstitial leukocytes were counted in 20 high-power fields, randomly chosen out of 40 cortical high-power fields per animal. Positive cells were counted per high-power field, omitting positive cells in glomerular fields.

### Light and Electron Microscopy

The lower half of the kidney was fixed in 4% buffered formalin, processed, and embedded in paraffin. Two-μm-thick sections were cut and stained with periodic acid-Schiff reagent. A small piece of cortical tissue from the lower

### Materials and Methods

#### Animals

Female inbred Balb/c mice (body weight, 18 to 21 g) were obtained from Charles River (Sulzfeld, Germany) and were kept in macrolone type III cages under a 12-h light/dark cycle. Water and standard chow (Sniff, Soest, Germany) were available ad libitum. For each time point, mice and controls that received apoferritin injections were kept in a single cage. Mice were killed by cervical dislocation. All experimental protocols were approved by the Animal Care and Ethics Committee of the Bavarian government.
kidney pole was fixed in glutaraldehyde and embedded in araldite for electron microscopic analysis. Slices that were cut with an ultramicrotome were stained with osmiumtetroxide and lead citrate. Electron microscopy was performed with a transmission electron microscope (Philips, Stuttgart, Germany).

**Immunofluorescence.** The upper kidney half was snap-frozen in liquid nitrogen, vacuum sealed, and stored at ~80°C. Two-μm-thick sections from the cut surface of the upper kidney half were analyzed. Glomerular IgG and HAP deposits were detected by a fluorescein-conjugated goat anti-mouse IgG antibody (1:250; Dianova, Hamburg, Germany), and an anti-horse ferritin antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. A nuclear counterstain with propidium-iodide (Sigma-Aldrich) was performed to localize glomeruli in controls. Thirty cortical glomeruli were assessed from each section. Glomeruli were within 50 μm from the capsule to avoid edge artifacts. Only positive glomerular signals were assessed by a semiquantitative score as follows: 0, background signal; +, low signal intensity; ++, moderate signal intensity; ++++, strong signal intensity.

**Immunohistochemistry.** Acetone-fixed frozen sections were air-dried and incubated in 3% hydrogen peroxidase to block endogenous peroxidases. Biotin was blocked applying the Vector Blocking Kit (Vector Laboratories, Burlingame, CA). Sections were washed in PBS and incubated with the primary antibody for 1 h at room temperature. The following rabbit and antibody couples were used as primary antibodies: anti-mCD45 (lymphocytes; Pharmingen, San Diego, CA; 1:200), anti-F4/80 (macrophages; Serotec, Oxford, UK; 1:50), anti-Mac-3 (macrophages; Pharmingen; 1:200), anti-CD3 (lymphocytes; Pharmingen; 1:100), anti Ki-67 (cell proliferation; Dianova; rabbit, 1:100), and anti-murine RANTES (Peprotech, Rocky Hill, NJ; 1:50). Anti-mouse MCP-1 antibodies were raised in rabbits using the peptide YIKNLDRNQMRSEPCT (murine MCP-1 amino acids 86 to 100 with an additional C at the carboxy terminus for coupling). The peptide was synthesized, conjugated to keyhole limpet hemocyanin as a carrier molecule, and used to immunize two rabbits followed by two booster injections given in 14-d intervals and two injections in 28-d intervals. The specificity of the antisera was demonstrated by a dot blot assay. Serial dilutions (1 μg, 100 ng, 10 ng, and 1 ng) of the peptide used for immunization and an unrelated peptide were spotted onto a nitrocellulose membrane and incubated with 1:1000 dilutions of the preimmune and immune serum. Bound antibodies were detected using a commercial enhanced chemiluminescence kit (Amersham, Freiburg, Germany) according to the manufacturer’s instructions (data not shown). For immunohistochemistry, a dilution of 1:50 was used. Rabbit preimmune serum served as a negative control. Signals of all primary antibodies were detected with a commercial mouse link and label kit following the instructions of the supplier (Biogenex SuperSensitive, San Ramon, CA). 3-Amino-9-ethylcarbazole substrate was used for signal development. All slices except for Ki-67 and Mac-3 staining were counterstained with hemalaun.

### In Situ Hybridization

Radiolabeled riboprobes containing mRANTES, mMCP-1, mCCR2, or mCCR5 cDNA fragments, flanked by RNA promoter sequences for SP6 and T7 RNA polymerases were used. The 321-bp mRANTES fragment represents positions 124 to 444 of the nucleotide sequence (GenBank accession number S37648). The 161-bp mMCP-1 fragment corresponds to nucleotides 299 to 459 (GenBank accession number J04467). The 153-bp mCCR2 fragment represents positions 1738 to 1888 of the nucleotide sequence (GenBank accession number U47035). The 220-bp mCCR5 fragment corresponds to nucleotides 1384 to 1604 (GenBank accession number D83648). Recombinant plasmids were used to transform *Escherichia coli* XL1-Blue. Plasmid DNA was prepared by alkaline lysis and purified over a cesium chloride gradient. The subcloned inserts were sequenced by the dideoxy-chain-termination method using a Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Weiterstadt, Germany) to confirm sequence identity. The constructs were linearized and used as templates for *in vitro* transcription by T7 and SP6 RNA polymerases (Boehringer Mannheim) generating antisense and sense probes, respectively. The probes were labeled with α-35S-UTP (1250 Ci/mmol; NEN, Cologne, Germany) to a specific activity of 8.3 × 10⁶ cpm/μg. Isotopic in situ hybridization with an α-35S-labeled RNA probes was performed according to modified standard protocols (20). Seven-μm frozen sections were treated with 0.2 N HCl at room temperature for 20 min for protein denaturation. Slides were rinsed in water and submerged for 30 min in prewarmed 2 % SSC at 70°C. After a short rinse in distilled water, slides were incubated with 5 μg/ml proteinase K in 50 mM Tris-HCl (pH 7.4) and 5 mM ethylenediaminetetraacetate (EDTA) for 10 min at room temperature. Sections were treated with glycine (0.2% in PBS) for 5 min and postfixed in 4% paraformaldehyde for 10 min at room temperature, followed by two washes in PBS. Sections were acetylated with 0.25% (vol/vol) acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 min, to avoid nonspecific binding of the probe. After the slides were rinsed twice in PBS, they were prehybridized in 50% formamide, 0.3 M NaCl, 10 mM sodium phosphate buffer (10 mM NaH₂PO₄, 10 mM Na₂HPO₄ [pH 6.8]), 10 mM Tris-HCl, 5 mM EDTA (pH 7.4), 10% dextran sulfate, 25 mM DTT, 1 × Denhardt’s, and 1.25 ng/ml tRNA at 52°C for 2 h. After prehybridization, slides were washed once in PBS and dehydrated in graded ethanol concentrations. The 35S-labeled antisense and sense RNA transcripts served as hybridization probe and control, respectively. Sections were hybridized at 52°C overnight with 12 μl of prehybridization solution containing labeled RNA probes with an activity of 1.0 × 10⁶ cpm per section, leading to a probe concentration of 0.3 ng/μl per kb (mRANTES probe) or 0.6 ng/μl per kb (mMCP-1 probe). After hybridization, four initial washes with 4 × SSC, 10 mM DTT (15 min, room temperature) were followed by a washing procedure with 50% formamide, 1 × SSC, 10 mM DTT at 50°C for 60 min. Sections were then incubated in a mixture of RNase A (40 μg/ml) and RNase T1 (50 U/ml) in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA [pH 7.4]) at 37°C for 30 min. They were washed three times in RNAse buffer, followed by two high-stringency washes in 0.1 × SSC, 10 mM β-mercaptoethanol (60°C, 15 min). Finally, slides were dehydrated in a graded series of ethanol containing 0.3 M ammonium acetate, 10 mM β-mercaptoethanol. For autoradiography, sections were dipped in Ilford K2 nuclear research emulsion diluted 1:1 with distilled water at 42°C and exposed for 2 wk at 4°C in a dry chamber. After development, tissue was counterstained with Harris hematoxylin and eosin.

### Real-Time Quantitative Reverse Transcription-PCR on Microdissected Renal Tissue

Glomeruli and tubulointerstitial specimen were microdissected manually and transferred to liquid nitrogen. Reverse transcription followed a protocol previously established for single glomerular podocytes (21). In brief, microdissected samples underwent random primed reverse transcription for 1 h at 42°C using a modified Moloney murine leukemia virus reverse transcriptase (Superscript; Life Technologies, Karlsruhe, Germany). Real-time reverse transcription-PCR (RT-PCR) was performed on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems) using a heat-activated TaqDNA poly-
merase (Amplitaq Gold, PE Biosystems). Thermal cycler conditions contained holds at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Messenger RNA expression for each signal was calculated following the ΔCt procedure (22). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as reference gene. The amplification efficiency of target and reference was shown to be similar, with a slope of log input amount to ΔCt, i.e., 0.0057 for CCR5 versus GAPDH. Controls consisting of ddH₂O were negative for target and housekeeper. The following oligonucleotide primers (300 nM) and probes (100 nM) were used: Murine CCR 5 (gb D 83648; bp 1065 to 1191): sense, 5'-CAAGACAACTCTGTGATGCTGCAA-3'; antisense, 5'-TCCTACTCCCAAGCTGCATAGAA-3'; internal fluorescence-labeled probe (FAM): 5'-TCTATACCCGATCCACAGGAGAACATGAAGTTT-3'; CCR5 specificity of primers and probe were tested on CCR plasmids; murine GAPDH (gb M32599; bp 730 to 836): sense, 5'-ATGCGCTGCTTCACCACCTTCT-3'; antisense, 5'-ATGCCTGCTTCACACCCCTTTC-3'; internal fluorescence labeled probe (VIC): 5'-CCAATGTGTCGTGTCGTGGATCTGA-3'. Primers and probes were obtained from PE Biosystems.

RNA Preparation and RNase Protection Assay from Kidneys

From each animal, the right kidney was snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was prepared using the method of Chomczynski and Sacchi (23). The final product was air dried, dissolved in dielaidoylphosphatedylcholine-treated water, and stored at −80°C.

Multiprobe template sets for mouse CC chemokines (mCK-5) and mouse CCR (mCR-5) for use in RNase protection assays were obtained from Pharmingen. Antisense riboprobes were prepared by in vitro transcription with T7 RNA polymerase (Promega, Madison, WI) and the incorporation of [32P]UTP at 37°C. Ten μg of total kidney RNA was used to analyze the expression pattern of CC chemokines, and 50 μg of RNA was used for analysis of CCR. Efficacy of RNase digestion was ensured by a yeast t-RNA sample in every assay. RNA samples were hybridized with 3 × 10⁶ counts of each [32P] UTP-labeled riboprobe for 14 h at 56°C. Free probe and other single-stranded RNA was digested with RNase A (Sigma) and T1 (Boehringer Mannheim) at 30°C for 30 min. RNases were digested with proteinase K (Promega). After phenol-chloroform extraction and sodium acetate-ethanol precipitation, the samples were electrophoresed through a denaturing 6% polyacrylamide gel. Gels were dried and exposed on phosphor screens of a Storm 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Bands were quantified using the ImageQuant software (Molecular Dynamics). Values are expressed relative to the GAPDH mRNA level in each lane after background subtraction.

Statistical Analysis

Data were expressed as means ± SEM and analyzed by t test for unpaired data. Statistical significance was defined as P < 0.05.

Results

Induction of GN

Albuminuria was increased significantly after 2 wk in mice that received HAF injections compared with controls (Up/Ucr, 140 ± 20 versus 17 ± 10; P < 0.01) but resolved after cessation of antigen injection (Figure 1). Body weight, serum creatinine, and blood urea nitrogen levels remained unchanged.

Figure 1. Course in mice that received horse apoferritin (HAF) injections (■) and controls (□). (A) Albuminuria as detected by mouse albumin enzyme-linked immunosorbent assay in spot urine samples, expressed as Uₐₐ/Urcreatinine (μg/mg). (B) Number of intraglomerular CD45-positive cells per glomerulus. (C) Levels of CC-chemokine mRNA related to respective glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA as detected by RNase protection assay; □, RANTES; △, monocyte chemoattractant protein-1 (MCP-1); ■, macrophage inflammatory protein-1β (MIP-1β). (D) Levels of CC-chemokine receptor (CCR) mRNA related to respective GAPDH mRNA as detected by RNase protection assay; □, CCR5; △, CCR2; ○, CCR1. Values are means ± SEM from four controls and 6 mice that received HAF injections at each time point for proteinuria and CD45-positive staining. RNA values were derived from three animals at each time point. * P < 0.05 versus wk 0.
Table 1. Serum, urinary, and histologic findings in HAF-induced glomerulonephritis

<table>
<thead>
<tr>
<th>Functional parameters</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 4</th>
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<tr>
<td></td>
<td>HAF</td>
<td>Controls</td>
<td>HAF</td>
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<tr>
<td>U₉₀/ₚ₉₀ (µg/mg)</td>
<td>20 ± 8</td>
<td>24 ± 10</td>
<td>140 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Scr (mg/dl)</td>
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<td>Sₘ₆₆ (mg/dl)</td>
<td>33 ± 5.6</td>
<td>26 ± 7.8</td>
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<td>body weight (g)</td>
<td>19 ± 1.7</td>
<td>19 ± 1.6</td>
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<td>Humoral response</td>
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<tr>
<td>anti-HAF IgG</td>
<td>52 ± 16&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4 ± 2</td>
<td>1536 ± 255&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>++</td>
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<tr>
<td>glomerular HAF deposits</td>
<td>++++</td>
<td>-</td>
<td>++/+++</td>
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<td>Cellular response</td>
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<td></td>
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<td>Ki-67+ (cells/glomerulus) leukocytes</td>
<td>2.4 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 ± 0.3</td>
<td>5.6 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>CD45+ intraglomerular</td>
<td>5.7 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>7.7 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>CD45+ periglomerular</td>
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<td>MAC3+ intraglomerular</td>
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<td>F4/80+ periglomerular</td>
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<td>F4/80+ interstitial</td>
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<td>0.8 ± 0.1</td>
<td>7.6 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup> Intra- and periglomerular cells were counted per glomerulus, interstitial cells were counted per high-power field (see Materials and Methods section). HAF, horse apoferritin; U₉₀/ₚ₉₀, urine albumin/urine creatinine ratio; Scr, serum creatinine; Sₘ₆₆, serum blood urea nitrogen. +, low signal intensity; ++, moderate signal intensity; ++++, strong signal intensity.

<sup>b</sup> P < 0.01 versus controls.

from prestudy values for mice that received HAF and saline injections at all time points. Anti-HAF titers were already elevated after 1 wk and increased further until week 4, i.e., after cessation of antigen injection in all mice that received HAF injections (Table 1).

**Histopathology**

Light microscopy revealed mild mesangial hypercellularity at week 1 (Figure 2). After 2 wk, diffuse mesangial expansion, with increase of matrix and mesangial hypercellularity, was observed in all animals that received HAF injections. Four wk after cessation of antigen injection, the histologic changes had returned to control levels. Immunohistochemical staining for the marker of cell proliferation Ki-67 revealed increased mesangial proliferation at weeks 1 and 2, which resolved at week 4 (Figure 3, Table 1). No endocapillary necrosis or crescents were noted. No overt tubulointerstitial lesions were noted at any time point. Kidneys of mice that received saline injections showed normal histology.

Mesangial deposits containing HAF and IgG were noted in mice that received HAF injections at all time points but not in controls (Figure 2). Semiquantitative assessment showed increasing mesangial HAF and IgG deposits from 1 to 2 wk and subsequent decrease after 4 wk (Table 1). Ultrastructurally electron-dense deposits were located in the mesangium and within the mesangial glomerular basement membrane (GBM) but not at the capillary loop GBM (Figure 4). Fusion of podocyte foot processes was not observed, which may be related to the moderate degree of proteinuria.

**Leukocytic Cell Infiltrate**

In mice that received HAF injections, infiltrating CD45-positive leukocytes were found within the glomerular tuft, periglomerulus, and cortical interstitium (Figure 3). However, there was a much stronger increase of the glomerular CD45-positive leukocyte cell count at week 2 compared with the interstitial compartment (which includes a much larger area compared with a single glomerular profile) (Table 1). The number of glomerular CD45-positive leukocytes and macrophages increased from week 1 to week 2 but had resolved at week 4 (Table 1). Glomerular Mac-3–positive macrophages were increased at week 2 (3.8 ± 0.9 versus 0.3 ± 0.2 in controls; P < 0.01; see Table 1, Figure 3), but CD3-positive lymphocytes were absent (data not shown). Because neutrophils and T cells were absent in glomeruli of mice that received HAF injections, the discrepancy of the CD45-positive and Mac-3–positive glomerular cell counts should be due to the fact that Mac-3 stains only a particular activation state of the monocyte-macrophage lineage.

**Chemokine Expression**

**RNase Protection Assays.** Total RNA was isolated from kidneys of 12 mice that received HAF injections and 6 control mice and analyzed by RNase protection assay. MCP-1 and...
RANTES mRNA expression was increased after 1 wk of antigen injection compared with saline injection (Figure 5A). Surprisingly, MCP-1 and RANTES mRNA expression had decreased toward control values at week 2, the time point of strong glomerular leukocyte infiltration and proteinuria (Figure 1). At week 4, when proteinuria and cell infiltrate had resolved, MCP-1 and RANTES mRNA expression were at control levels. Expression of eotaxin, MIP-1α, MIP-1β, MIP-2, T-cell activation gene-3, and interferon-inducible protein-10 were not increased when related to GAPDH levels at any time point.

In Situ Hybridization. To localize the sites of chemokine expression, we performed in situ hybridization on frozen kidney sections with antisense probes for RANTES and MCP-1, the two upregulated chemokines. For both MCP-1 and RANTES, a positive staining was found only after 1 wk in mice that received HAF injections (Figure 6). Positive cells were detected exclusively within but not outside the glomeruli. Hybridization with sense probes was negative as were tissues from controls that received saline injections.

Immunohistochemistry. Consistent with the in situ hybridization data, staining with antibodies against MCP-1 and RANTES revealed positive staining of only intraglomerular cells in mice that received HAF injections at week 1 (Figure 6). MCP-1 and RANTES were not detected at later time points or in glomeruli of mice that received saline injections. The location of positive cells was similar to that found by the respective in situ hybridization. Immunohistologic staining with rabbit preimmune serum was negative.

CCR Expression

RNase Protection Assays. RNase protection assays with RNA from kidneys of mice that received saline injections revealed no signals for any of the CCR tested (CCR1 to CCR5) despite strong signals for GAPDH and L32 (Figure 5B). In kidneys of mice that received HAF injections, elevated levels were observed for CCR1, CCR2, and CCR5 at 1 and 2 wk. No signals for CCR3 and CCR4 were detectable. Expression of CCR1 was elevated threefold at week 1, decreased to twofold at week 2, and had returned to the low levels of the controls.
that received saline injections at week 4 (Figure 1). Levels of mRNA for CCR2 were elevated threefold at week 1 and decreased at weeks 2 and 4 to twofold and 1.5-fold of controls, respectively. Similarly, mRNA of CCR5 was elevated fourfold at week 1 and decreased to twofold of controls.

**Real-Time Quantitative RT-PCR.** To localize better the CCR expression, RT-PCR of microdissected glomeruli and tubulointerstitial specimens and *in situ* hybridization were performed. The glomerular mRNA expression of CCR5 was fourfold higher in mice that received HAF injections compared with that of controls that received saline injections (*P* < 0.01; Figure 7). Comparison of tubulointerstitial specimens between mice that received HAF and saline injections revealed no difference in CCR5 expression. The signal for CCR2 was too weak for quantitative analysis. RT-PCR was not conducted for CCR1.

**In Situ Hybridization.** Hybridization with an antisense probe for CCR5 revealed a positive staining for CCR5 in mice that received HAF injections but not saline injections at week 1 or hybridization with sense probes (Figure 8). Only in glomeruli were single cells positive for CCR5. No CCR5-positive cells could be detected in the tubulointerstitial space, confirming the results obtained by RT-PCR. Also in agreement with the RT-PCR data, no consistent signal for CCR2 could be obtained by *in situ* hybridization.

**Discussion**

The present data show that in a model of murine IC-GN with glomerular macrophage accumulation and mesangial cell proliferation but without significant tubulointerstitial injury, the expression of chemokines and CCR is restricted to the glomerulus. The generation of MCP-1 and RANTES and the expression of their respective receptors CCR1, CCR2, and CCR5 precedes proteinuria and the maximum of leukocyte infiltration and is already downregulated during active and resolved IC-GN. These results argue for an important role of chemokines and CCR in the early initiation phase of glomerular leukocyte infiltration and for a lesser role during the phase with persistent

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*Figure 3. Histopathology of HAF-GN at week 2. Glomerular CD45-positive cell infiltrate in HAF-GN (A), absent in control mice (B). Accumulation of glomerular Mac-3–positive macrophages in mice that received HAF injections (C, arrows) but not in mice that received saline injections (D). Glomerular Ki-67–positive cells indicate proliferative GN (E, arrows) compared with controls that received saline injections (F). Magnifications: ×1000 in A and B; ×400 in C, D, E, and F.*
leukocyte infiltration and proteinuria at the height of the
disease.

HAF-Induced GN Is a Reversible, Proliferative Disease
Restricted to the Glomerulus

A 14-d course of HAF injection into Balb/c mice resulted in
IC-GN with glomerular macrophage infiltration, mesangial
proliferation, proteinuria, and the absence of major tubulointerstitial inflammation or damage. Glomerular changes of this
IC-GN model were induced by deposition of immune comple-
exes consisting of the injected antigen and autologous Ig at
the paramesangial GBM and the mesangium. When the antigen
administration was stopped, the proliferative changes, leuko-
cyte infiltration, and proteinuria resolved within a 2-wk inter-
val and the normal structure of the glomerular tuft was re-
stored. This resolution occurred despite persistently elevated
anti-HAF serum antibody titers and only slightly reduced glo-
merular IC deposits, as judged by immunofluorescence. Thus,
to generate a glomerular inflammatory response, a continuous
de novo intraglomerular IC deposition seems to be required.

This feature of IC trailing the resolution of the proliferative GN
is also reminiscent of some human IC-GN, such as postinfectious GN or IgA nephropathy (24). Thus, the histology of
HAF-GN bears close similarities to human IC-GN, such as
postinfectious GN, lupus nephritis class IIb, cryoglobulin-re-
lated GN type II, and other forms of mesangioproliferative
IC-GN. The course of initiation and resolution resembles in
particular postinfectious GN or IgA nephropathy. Furthermore,
HAF-GN shows almost exclusively glomerular lesions with
very little interstitial cell infiltration or damage. This allows the
study of the role of mediators for predominant glomerular
lesions during the initiation and resolution phases of glomer-
ular IC nephritis without major interference by tubulointerstitial disease.

Early MCP-1 and RANTES Expression Is
Downregulated already at the Height of GN

We used the course of HAF-induced GN to study chemokine
and CCR expression during evolution and resolution of a
glomerular leukocytic infiltrate. The finding of MCP-1 and
RANTES expression early in the course of GN is in concordance with results reported by previous studies of murine NSN as well as in human GN (5, 8, 25–27). Surprisingly, we found that glomerular MCP-1 and RANTES expression is already completely downregulated at the peak of glomerular leukocyte infiltration and the onset of proteinuria. This argues for downmodulation of local chemokine generation despite persistent IC deposition and against a self-maintaining amplification loop of chemokine production by infiltrating leukocytes. To the contrary, locally generated “antagonists” might inhibit further chemokine expression. Potential candidates for a counterregulatory local suppression of chemokine production are, e.g., transforming growth factor-β1, nitric oxide, prostaglandin E1, and other yet unknown factors (28–32).

Furthermore, we could demonstrate that in evolving GN, MCP-1 and RANTES are expressed by some but not all glomerular cells. As judged from the in situ hybridization and immunohistology, these could be endothelial cells, mesangial cells, and infiltrating monocytes but not podocytes. Because Tesch et al. (8) observed tubular and interstitial but not glomerular MCP-1 expression in a model of predominantly tubulointerstitial leukocyte infiltration, the present data indicate that depending on the disease model, MCP-1 expression is localized to the predominant site of inflammation.

In view of the many chemokines known, the limited repertoire of CC chemokines that are found to be upregulated during in vivo studies is striking. Predominantly, MCP-1 and RANTES among many others examined have been reported in various models and in human renal disease (2). RANTES is known to attract T cells and monocytes in vitro, as well as in the spontaneous nephritis of MRL-Fas(1pr) mice, in experimental murine NSN, and during renal transplant rejection in rats (8, 10, 33). Interestingly, the marked RANTES expression in HAF-GN was not associated with lymphocyte infiltration. This observation supports the hypothesis that the monotonous pattern of chemokine expression unfolds its selectivity locally, perhaps by selective CCR and adhesion molecule expression on restricted target cells (2).

**CCR Expression also Precedes the Peak of HAF-GN Activity**

Chemokines attract leukocytes by binding and activating specific CCR on subclasses of leukocytes. Therefore, it was interesting to find that indeed CCR1, CCR2, and CCR5 mRNA
expression was upregulated together with their respective ligands MCP-1 and RANTES early in the course of GN. In HAF-GN, CCR2 and CCR5 mRNA expression originates from glomeruli, most likely from their presence on infiltrating macrophages by analysis of isolated glomeruli by RT-PCR and in situ hybridization of renal sections. The peak of chemokine and CCR levels preceded the maximum leukocyte infiltration during the course of disease. At 2 wk, when MCP-1 and RANTES mRNA levels had returned to normal, CCR mRNA levels remained elevated, consistent with the glomerular leukocytic cell infiltrate. We and others recently described a similar upregulation of CCR1, CCR2, and CCR5 in cortical and glomerular preparations of NSN (5,16). In various human renal diseases, CCR5 is found only on infiltrating leukocytes, mostly T cells and monocyte/macrophages (13–15). With the exception of CXCR3, CCR have not been
found on intrinsic renal cells, in both normal and diseased human kidneys (34).

CCR1 (receptor for RANTES and MIP-1α) is expressed on neutrophils, monocytes, and activated T cells (35). As recently shown by us (36), it can also be induced in human mesangial cells upon stimulation with interferon-γ. Therefore, during early HAF-GN, mesangial cells might contribute to the increase of CCR1 mRNA expression. Once activated, CCR1 is rapidly downregulated on effector cells, which is supported by our finding that CCR1 mRNA expression precedes the maximum of leukocyte infiltration in GN (37). CCR1 may also play a role in regulating the immune response in addition to its chemoattractive properties. For example, a recent report noted a worsening of NSN in CCR1-deficient mice in comparison to wild-type mice (16). This was related to an enhanced Th1 response and a counterregulatory role of CCR1. Unfortunately, we could not localize further the CCR expression to specific cell types in HAF-GN. Therefore, future work will have to identify these cells and their role in IC-GN.

CCR2, the only known receptor for murine MCP-1, is expressed on monocytes, macrophages, activated T cells, and activated endothelial cells (38,39). Apart from mediating chemoattractive effects of MCP-1 on circulating leukocytes, CCR2-positive cells seem to enhance directly Th1-type immune responses, e.g., crescentic GN, by increased expression of interferon-γ (40). In murine crescentic GN, CCR2 transcripts have been detected in glomerular isolates by RT-PCR. Also in NSN, CCR2-deficient mice exhibit reduced infiltration of macrophages and proteinuria despite aggravated histopathologic damage (5,41). Declining CCR2 mRNA expression during the maximum of leukocyte infiltration in HAF-GN may be a consequence of receptor downregulation during the differentiation of monocytes to tissue macrophages, a known feedback mechanism in the regulation of the chemotactic response of monocytes/macrophages (42). Unfortunately, the signal for CCR2 was too weak to allow further localization by RT-PCR or in situ hybridization in HAF-GN.

CCR5 (receptor for MIP-1α, MIP-1β, and RANTES) can be expressed on monocytes, activated T cells, and natural killer cells. In human allograft rejection as well as in a variety of other glomerular and tubulointerstitial diseases, CCR5 was detected only on infiltrating cells, predominantly T cells and macrophages, but not on intrinsic renal cells (13,14). In this model of IC-GN with predominant glomerular macrophage infiltration, the expression of CCR5 was also localized to the glomerulus by both RT-PCR and in situ hybridization. As shown in Figure 8, only single cells within a glomerular section are positive. This argues in favor of the CCR5-positive cells’ being infiltrating leukocytes, as positivity of intrinsic glomerular cells, e.g., endothelial, mesangial, or epithelial cells, should have resulted in multiple signals per glomerular section. Unfortunately, this issue cannot be resolved at present because of a lack of appropriate reagents. Of special interest is the decrease of CCR5 expression during active IC-GN despite persistent leukocyte infiltration. Most likely, this relates to receptor downregulation on the respective target cells after binding of the chemokine ligand (43). Furthermore, additional humoral factors such as prostaglandin E2 may contribute to rapid CCR5 downregulation (44). When antigen injection and hence de novo immune complex deposition are stopped, chemokine and CCR expression decreased further within 14 d. This occurred in parallel with the resolution of mesangial proliferation, leukocyte infiltration, and proteinuria. Presumably, the lack of further chemokine secretion as well as CCR downregulation results in decreased chemotaxis and adherence of leukocytes. The resolution of glomerular proliferation may be a result of cell emigration and/or cellular apoptosis (45).

In summary, we describe a murine model of transient IC-GN, which is characterized by mesangial proliferation and glomerular macrophage infiltration in the absence of major tubulointerstitial disease. During the course of IC-GN, the early glomerular expression of MCP-1 and RANTES precedes
proteinuria and is already downregulated at the maximum of glomerular leukocyte infiltration. Their respective receptors CCR1, CCR2, and CCR5 are also upregulated early together with the leukocyte infiltration. Resolution of IC-GN is associated with a decline of chemokine and CCR mRNA levels. These results suggest a role for MCP-1 and RANTES as initial chemotactic signals attracting CCR1-, CCR2-, and CCR5-positive leukocytes. Overexpression of MCP-1 and RANTES seems not to be necessary for the persistence of leukocyte infiltration and the resulting proteinuria at the height of glomerular injury.

Acknowledgments

The authors thank Y. Linde and E. Göckel-Krzikalla for their technical assistance. The data have been reported previously in abstract form (J Am Soc Nephrol 10: A2652, 1999). The work was supported by grants from the German Human Genome Project and the Else-Kroener-Fresenius Foundation (M.K.), from the University of Munich (H.J.A.), and from the Deutsche Forschungsgemeinschaft (B.L.).

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