Chemokine Expression Precedes Inflammatory Cell Infiltration and Chemokine Receptor and Cytokine Expression during the Initiation of Murine Lupus Nephritis

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Abstract. Lupus nephritis is characterized by immune complex deposition and inflammatory cell infiltration. Therefore, the initiation and progression of lupus nephritis in MRL/MpJ Fas lpr/lpr (MRL/lpr) mice were investigated, with a focus on the expression of several chemokines and chemokine receptors. Mice were monitored for proteinuria from 6 to 20 wk of age, and kidneys were examined every 2 wk by light microscopy, electron microscopy, and immunohistologic analyses. Furthermore, the expression of chemokines, chemokine receptors, and proinflammatory cytokines was analyzed in ribonuclease protection assays. MRL/lpr mice demonstrated increased expression of monocyte chemoattractant protein-1, regulated upon activation, normal T cell-expressed and -secreted protein, inducible protein of 10 kD, and macrophage inflammatory protein-1β at week 8. At that time point, levels of circulating and glomerular immune complexes were increased, and no proteinuria or histopathologic signs of renal damage could be observed. As assessed in immunohistochemical and in situ hybridization analyses, monocyte chemoattractant protein-1 and regulated upon activation, normal T cell-expressed and -secreted protein expression was preferentially located in the glomeruli and interstitium. Mononuclear cell infiltration of the kidney was observed by weeks 10 to 12. At week 12, the renal expression of chemokine receptor 1 (CCR1), CCR2, and CCR5 was increased, mice became proteinuric, and renal damage was histologically evident. Finally, the expression of proinflammatory cytokines was detected (weeks 12 to 14). In summary, (1) chemokines are upregulated before inflammatory cell infiltration, proteinuria, and kidney damage are observed; (2) chemokine generation is restricted to sites of subsequent inflammatory cell infiltration, i.e., glomeruli and interstitium; (3) chemokine receptor expression parallels mononuclear cell infiltration; and (4) proinflammatory cytokines are upregulated later, in parallel with inflammatory cell infiltration and the onset of proteinuria. These results support the hypothesis that chemokines initiate leukocyte infiltration and precede proteinuria and renal damage in MRL/lpr mice.
vival, for MRL/lpr mice rendered genetically deficient for MCP-1, compared with wild-type or heterozygous lupus mice. Because the renal influx of inflammatory cells was not totally suppressed and because multiple chemokines usually act in concert, additional chemokines may be involved. Interestingly, our laboratory recently described a prominent periglomerular and interstitial mononuclear cell infiltrate positive for chemokine receptor 5 (CCR5); a main chemokine receptor for regulated upon activation, normal T cell-expressed and -secreted (RANTES) protein, macrophage inflammatory protein-1α [MIP-1α], and MIP-1β) in biopsies from patients with lupus nephritis (21). Although it is apparent that chemokines contribute significantly to leukocyte influx into sites of tissue injury, they must be considered part of a concerted interaction involving cytokines, adhesion molecules, and other mediators of inflammation. For example, proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) stimulate renal cells to produce chemokines in vitro (22–24), whereas other cytokines, such as transforming growth factor-β (TGF-β), can suppress their synthesis (25). Furthermore, TGF-β has been implicated in the progressive fibrosis of renal disease. There is experimental evidence that TNF-α, IL-1β, and IFN-γ play important pathogenic roles in lupus nephritis (26–28).

However, little is currently known regarding the temporal sequence of the intrarenal generation of chemokines and cytokines or their relationships to the inflammatory cell infiltrates, the generation of proteinuria, and the resulting renal pathologic processes and functional insufficiency. Knowledge of this immune pathologic network and its temporal regulation is an important requirement for evaluation of the potential pathophysiologic roles of chemokines and cytokines during lupus nephritis and for subsequent design of therapeutic strategies to interfere with their actions. For example, does cytokine generation precede that of chemokines or vice versa, and how do these factors relate to the inflammatory cell infiltrate?

To address these questions, we systematically characterized the expression of a series of chemokines and chemokine receptors, as well as inflammatory cytokines, during the initiation and progression of lupus nephritis in MRL/lpr mice. Our results demonstrate that the intrarenal generation of chemokines precedes cellular infiltration, chemokine receptor expression, and proinflammatory cytokine production, as well as the resulting proteinuria and functional renal impairment. Therefore, chemokines may play an important role in the initiation and further development of lupus nephritis.

Materials and Methods

Reagents and Antibodies

A custom antipeptide antiserum against murine MCP-1 was raised in rabbits, as described previously (29). A FITC-conjugated rabbit anti-mouse Ig antibody specific for both IgG and IgM (IgG+M) (Boehringer Mannheim, Mannheim, Germany), a rabbit anti-mouse RANTES polyclonal antibody (PeproTech, London, UK), and a peroxidase-conjugated goat anti-rabbit Ig secondary antibody (Dako, Glostrup, Denmark) were used. The peroxidase-conjugated CD3 monoclonal antibody was from Serotec (Oxford, UK). All reagents for the anti-DNA enzyme-linked immunosorbent assay (ELISA) were from Boehringer Mannheim, except the mouse monoclonal anti-single- and double-stranded DNA antibody used as a standard (Chemicon International, Temecula, CA). Radiolabeled [α-35S]UTP (1.25 Ci/mmol) and [α-32P]UTP (3000 Ci/mmol) were from NEN (Boston, MA). All other reagents or solvents (analysis grade) were from Merck (Darmstadt, Germany). The multiprobe template sets (mCK2b, mCK3, mCK5, and mCR5) for analysis of mRNA expression using ribonuclease protection assays (RPA) were from Pharmingen (San Diego, CA).

Experimental Design

MRL and MRL/lpr mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed under specific pathogen-free conditions, with free access to water and food. All experimental procedures were performed according to German law (Tierschutzgesetz). Permission was given by the local governmental authorities (211-2531.3-11/99).

Mice were distributed into different experimental groups, each consisting of three to six female mice. Beginning at the age of 6 wk, groups of MRL (control) and MRL/lpr (lupus) mice were euthanized every 2 wk. Spot urine samples were obtained immediately before euthanasia, and blood samples were obtained by cardiac puncture after cervical dislocation. The following parameters were determined using standard analytical protocols: Bradford assay (30) (microtiter scale) for proteinuria assessments (BioRad, Richmond, CA), Jaffe method (31) for creatinine measurements (Merck Diagnostika), urease/glutamate dehydrogenase method (32) for blood urea nitrogen measurements (Merck Diagnostika), and IgG ELISA for analysis of circulating IC (Boehringer Mannheim) and anti-double-stranded DNA antibodies (33).

One kidney was used exclusively for isolation of total RNA, using a standard protocol (34). The upper pole of the other kidney was used for ultrastructural study. The remaining part of the kidney was split into two halves. One half was fixed in formaldehyde for routine histologic examination and in situ hybridization. The other half of the renal tissue was embedded in OCT medium (Jung; Leica Instruments, Wetzlar, Germany), snap-frozen in liquid nitrogen, and stored at −80°C until used for immunohistochemical analysis.

Renal Morphologic Analyses

Light Microscopy. Renal tissue was fixed in neutral buffered formaldehyde in saline, and 3-μm, paraffin-embedded sections were stained with hematoxylin/eosin and period acid-Schiff stain. The severity of the renal lesions was graded as 0 to 3+, using the activity index described for human lupus nephritis by Austin et al. (35).

Electron Microscopy. For ultrastructural studies, the kidney tissue was immediately fixed for 2 h in cacodylate buffer containing 2.5% glutaraldehyde, followed by postfixation in osmium tetroxide. Tissue was then dehydrated in increasing concentrations of ethanol and embedded in Epon 812 (Merck). Ultrathin sections were stained with lead citrate and examined at 75 kV using a JEOL CX electron microscope (JEOL, Tokyo, Japan) (36).

In Situ Hybridization

In situ hybridization with radiolabeled cRNA probes was performed on 4-μm sections of paraffin-embedded tissues, as described previously (29). Briefly, section slides were prehybridized at 52°C for 2 h. After prehybridization, slides were washed and dehydrated with graded ethanol concentrations. Fragments of murine MCP-1 (positions 299 to 459, GenBank database accession number J04467),
Renal mRNA Expression

Cytokine, chemokine, and chemokine receptor expression was analyzed by RPA. Twenty micrograms of total RNA from each sample was used for the cytokine and chemokine expression analyses by multiprobe RPA (RiboQuant mCK2h, mCK3, and mCK5 template sets; Pharmingen). Fifty micrograms of RNA was used for the chemokine receptor template set mCR5. Assays were performed according to the instructions provided by the manufacturer. The RNase-protected probes were separated on 5% denaturing polyacrylamide gels and analyzed by phosphorimaging (Storm 840 PhosphorImager; Molecular Dynamics, Sunnyvale, CA). Bands were quantified using ImageQuant software (Molecular Dynamics, Eugene, OR). In preliminary experiments, the identities of the various templates in the multiprobe RPA kits were confirmed by sequencing.

Statistical Analyses

Data were expressed as means ± SD and analyzed with either the unpaired two-way t test (for parametric data) or the Mann-Whitney U test (for nonparametric data). The null hypothesis was rejected at \( P < 0.05 \).

Results

Biochemical Analyses of Serum Parameters

MRL/lpr (lupus) mice demonstrated characteristic alterations of serum immune parameters, with progressive increases in the levels of circulating IC (Figure 1A) and anti-DNA antibodies (Figure 1B) with age, compared with age-matched MRL (control) mice. For MRL/lpr mice, circulating IC levels were observed to be elevated as early as 8 wk of age \((n = 3)\) and increased progressively until they reached a maximum at week 16 \((n = 6)\). Plasma levels of anti-DNA antibodies started to significantly increase at 10 wk of age \((n = 3)\) and demonstrated a marked additional increase at 12 to 20 wk of age \((n = 3 \text{ to } 6 \text{ animals/time point})\). No changes with age occurred in the MRL control mice.

The renal function of MRL/lpr mice was compromised, as demonstrated by increases in proteinuria (Figure 1C) and plasma urea levels (Figure 1D). Both parameters increased significantly at somewhat later time points (weeks 12 to 14) and continuously increased until week 20 \((n = 3 \text{ to } 6)\). In contrast, proteinuria was absent and blood urea nitrogen levels were unaltered in control animals throughout the study period.

**Histopathologic Analyses**

**Renal Morphologic Features.** MRL/lpr mice demonstrate progressive development of renal damage, which is noticeable at 10 wk of age and reaches significance at 12 wk. Figure 2 (A to D) presents representative photographs of hematoxylin/eosin-stained renal tissue from lupus mice \((n = 3 \text{ to } 6)\) of 8, 10, 12, or 14 wk of age. The observed lesions included the presence of enlarged hypercellular glomeruli, with increased numbers of both resident cells and infiltrating leukocytes. A parallel increase in mesangial matrix was also noted. Prominent interstitial mononuclear cell infiltrates were also observed, with predominantly perivascular localization in the cortex and medulla of the affected kidneys. Occasionally, focal glomerular capillary thrombi, fibrinoid necrosis, and crescents were identified in later phases.

The renal histologic lesions of the MRL/lpr mice exhibited progressively increasing activity indices, which began to be significant at 12 wk of age (Figure 1E). The activity indices increased with age for lupus mice, so that at the age of 20 wk all mice exhibited signs of severe renal damage \((n = 3)\). In contrast, no histopathologic abnormalities could be observed in MRL mice at any time point \((n = 3 \text{ to } 6)\).

**Direct Immunofluorescence Analyses.** MRL/lpr mice (10 to 14 wk of age) exhibited generalized, diffuse, granular, irregularly distributed mesangial IC deposits \((n = 3 \text{ to } 6)\) (Figure 2H). In addition, positive antinuclear antibody staining was observed in some 14-wk-old animals (data not shown). Eight-week-old mice demonstrated no consistent staining for IC \((n = 3)\) (Figure 2F).

**Ultrastructural Studies.** Ultrastructural analyses using transmission electron microscopy were performed for two lupus mice and one control mouse at 8, 10, and 14 wk of age. Eight-week-old mice demonstrated no ultrastructural alterations (Figure 2E). In contrast, 10-wk-old mice (Figure 2G) exhibited hypercellular glomeruli with mesangial matrix expansion and edema, in addition to well defined, electron-dense deposits. The most consistent ultrastructural finding at 14 wk of age was the presence of prominent electron-dense deposits localized preferentially in the mesangium and in the subendothelial area of the glomeruli. Occasionally, small electron-
dense deposits were also identified in the glomerular subepithelium. Focal podocyte foot process effacement and endothelial cell edema, with capillary lumen obliteration, were also observed. No significant ultrastructural alterations were noted in the control groups at any of the time points examined.

**Renal Chemokine Expression**

**Renal Chemokine mRNA Expression.** Chemokine mRNA expression was analyzed by RPA of total kidney RNA. As shown in Figure 3, lupus mice exhibited significant increases in renal mRNA for the chemokines MCP-1, RANTES, interferon γ-inducible protein 10 (IP-10), and MIP-1β, starting at 8 wk of age. Other chemokines examined (lymphotactin, eotaxin, MIP-1α, MIP-2, and T-cell activation protein 3) were undetectable. MRL/lpr mice exhibited progressive increases in chemokine expression from week 8 to week 16 (n = 3 to 6/time point), compared with the age-matched MRL control animals (n = 3). The barely

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**Figure 1.** Time course of renal disease in MRL and MRL/MpJ Fas<sup>−/−</sup> (MRL/lpr) mice. (A) Circulating immune complex (IC) levels, as quantified in plasma samples with an enzyme-linked immunosorbent assay (ELISA) for mouse IgG M. (B) Anti-DNA antibody levels, as quantified in plasma samples with an ELISA. (C) Proteinuria, as detected in spot urine samples by the Bradford method, expressed as the urinary protein/creatinine ratio. (D) Blood urea nitrogen levels, as measured in plasma samples using the urease/GIDH method. (E) Histologic renal lesions (activity index), scored as previously described by Austin et al. (35) for human patients with lupus nephritis. Values are means ± SD for three to six animals in each group (*, P < 0.05 versus respective time-matched control).
detectable chemokine expression in the MRL control mice remained unaltered during the study period.

It should be noted that a polymorphism in the murine IP-10 gene can lead to false-negative results with the mCK5 RPA multiprobe kit (38). The results for IP-10 were therefore confirmed by using single-template RPA (data not shown).

**Immunohistochemical Analyses.** To localize the major CC chemokines detected, i.e., MCP-1 and RANTES, immunohistologic staining of renal tissue was performed. Figure 4 presents representative examples of MCP-1 and RANTES staining in 14-wk-old MRL mice (n = 3) (Figure 4, A and E) and MRL/lpr mice (n = 2) (Figure 4, B and F). Immunohistologic analyses of both chemokines demonstrated marked positivity within the glomeruli of the MRL/lpr mice. The staining for both chemokines was stronger in the glomerular areas that also demonstrated increased mononuclear cell accumulation. Positive staining was also observed in the mesangial area. This expression pattern was consistent at 12 wk of age (n = 2) and increased until week 16 (n = 2). Despite the increasing intensity of the immunohistologic signals for

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**Figure 2.** Histopathologic kinetics of glomerular and tubulointerstitial damage in MRL/lpr mice. (A to D) Light microscopic microphotographs of hematoxylin/eosin-stained sections from representative mice of different ages. Lesions progressively increased with the age of the animals, from an absence of lesions (8 wk) (A) to initial (10 wk) (B), mild (12 wk) (C), and well established (14 wk) (D) lesions. (F and H) Representative direct immunofluorescence micrographs, showing negative staining of a sample from an 8-wk-old MRL/lpr mouse (F) and generalized, diffuse, irregular, granular staining, corresponding to mesangial IC deposits, in a sample from a 10-wk-old MRL/lpr mouse (H). (E and G) Ultrastructural microphotographs of representative 8-wk-old (E) and 10-wk-old (G) MRL/lpr mice. No electron-dense deposits could be detected at the age of 8 wk, whereas mice exhibited numerous deposits (arrows) at 10 wk. Magnifications: ×400 in A through D; ×9900 in E and G; ×200 in F and H.
MCP-1 and RANTES, the tissue distributions remained unaltered throughout the study period for the MRL/lpr mice. Some patchy tubulointerstitial MCP-1 and RANTES staining was occasionally noted. Finally, positive tubular staining localized to the brush border or the tubular lumen was also occasionally observed. No specific MCP-1 or RANTES staining was noted in samples from control MRL mice at any of the time points or in MRL or MRL/lpr samples stained with preimmune serum (data not shown).

In Situ Hybridization. To further localize the sites of MCP-1 and RANTES production, in situ hybridization analyses were performed. With this method, MCP-1 and RANTES mRNA was observed within the glomeruli and tubulointerstitium. Figure 4 (C and D) presents the in situ hybridization results for MCP-1 mRNA expression in MRL (n = 1) and MRL/lpr (n = 2) mice. Figure 4 (G and H) demonstrates the corresponding RANTES expression. Occasionally, signals for either chemokine could be observed in the tubules or in the interstitial inflammatory cell infiltrate. Therefore, the in situ localization data corroborate the immunohistologic localization of MCP-1 and RANTES predominantly in the glomeruli and in some tubulointerstitial areas. No consistent signals could be detected with sense riboprobes.
Renal Chemokine Receptor Expression

Renal Chemokine Receptor mRNA Expression. By RPA, CCR2 and CCR5 were noted to be upregulated in MRL/lpr mice (Figure 5). CCR2 and CCR5 expression increased beginning at week 12 (CCR2, twofold; CCR5, 3.5-fold; \(n = 3\)) and progressively increased until week 16 (CCR2, ninefold; CCR5, 12-fold; \(n = 6\)). The constitutive expression of CCR1 increased with age in both control MRL and lupus MRL/lpr kidneys. Later, a consistent difference in renal CCR1 expression could be observed between the two groups of mice. A threefold increase in CCR1 mRNA levels at week 14 (\(n = 6\)) and a fourfold increase at week 16 (\(n = 6\)) were detected in MRL/lpr kidneys, compared with MRL control kidneys (\(n = 3\)).

In Situ Hybridization. For localization of CCR2 and CCR5 mRNA, in situ hybridization analyses were per-
formed for each gene in kidneys from 14-wk-old MRL/lpr ($n = 2$) or MRL ($n = 2$) mice. As shown in Figure 6, MRL/lpr mice demonstrated slight tubulointerstitial signals for both chemokine receptors, i.e., CCR5 (Figure 6A) and CCR2 (Figure 6B). The signals co-localized with the interstitial cell infiltrate and were occasionally detected within hypercellular glomeruli and in periglomerular mononuclear cell infiltrates, but no mRNA expression could be detected in tubular cells. CCR2 was mainly localized to mononuclear cells that were CD3-negative (Figure 6D), whereas CCR5 was primarily expressed on CD3-positive lymphocytes (Figure 6C). No signals could be detected with the sense probes (data not shown).

Proinflammatory Cytokine Expression

Renal mRNA Expression. Because several cytokines seem to play a role in lupus nephritis, proinflammatory cytokine mRNA expression was analyzed by multiprobe RPA

Figure 5. Time course of renal chemokine receptor mRNA expression. Fifty micrograms of total RNA isolated from whole kidney was analyzed using the mCR5 RPA kit from Pharmingen. (A) Representative RPA gel. Samples were organized in time groups, and each group was separated from the others by an empty lane. For each group, samples were loaded on the gel using the following scheme: first lane, MRL control sample (M); in the next three lanes, samples from three different MRL/lpr mice (ML). T. tRNA control sample (50 μg). Expression of chemokine receptor 5 (CCR5) and CCR2 was increased in MRL/lpr mice, compared with the respective control animals, beginning at weeks 10 to 12. CCR1 expression was upregulated beginning at week 14. (B and C) Densitometric analyses of CCR5 (B) and CCR2 (C) expression.
using the mCK2b and mCK3 template sets. Kidneys from lupus mice exhibited significant increases in mRNA expression levels for IL-1β and its corresponding negative regulator IL-1 receptor antagonist (Figure 7A), beginning at weeks 12 to 14. In addition, MRL/lpr mice exhibited higher levels of lymphotixin-β, TNF-α, IFN-γ, and IFN-β at weeks 10 to 12 (Figure 7B). The observed upregulation of TNF-α, IFN-γ, and IFN-β expression at week 10 preceded the upregulation of lymphotixin-β and IL-1β observed in lupus mice at week 12.

TGF-β has been proposed to mediate the progression of fibrosis in renal diseases. Interestingly, the renal mRNA levels for this cytokine increased equally with age in MRL/lpr mice and MRL control mice. This finding was noted for both isoforms examined, i.e., TGF-β1 and TGF-β2.

**Discussion**

In this study, we systematically characterized the expression of a series of chemokines, proinflammatory cytokines, and chemokine receptors during the initiation and progression of lupus nephritis in MRL/lpr mice. Our results indicate that (1) renal chemokine expression is upregulated in parallel with IC deposition but upregulation occurs before cellular infiltration, proteinuria, and kidney damage; (2) the expression of chemokines is restricted to glomeruli and the interstitium, i.e., the sites of cellular infiltration; and (3) the proinflammatory cytokines are upregulated in parallel with inflammatory cell infiltration and the associated expression of chemokine receptors but after the upregulation of chemokines. The time course of the events places the generation of chemokines at a very early point in the course of lupus nephritis, consistent with their proposed role as early mediators of renal inflammation.

The expression of some chemokines has been linked to lupus nephritis in patients and in animal models of the disease. Biopsies from patients with lupus nephritis have demonstrated the presence of the chemokine MCP-1 in endothelial, monocytic, and cortical tubular epithelial cells (16,17). Several authors reported increased MCP-1 urinary excretion by patients with SLE (17,18,39). The elevated urinary MCP-1 excretion reflected lupus nephritis disease activity. Zoja et al. (18), with NZB/W mice, and Tesch et al. (19), with MRL/lpr mice, observed increased renal MCP-1 expression before the onset of proteinuria. Our results confirm that proteinuria occurs after 12 wk of age, whereas chemokine expression is already upregulated at 8 wk of age. In MRL/lpr mice made genetically deficient for MCP-1, Tesch et al. (19) observed a marked decrease in macrophage and T cell recruitment, with concordant reductions in kidney pathologic features and proteinuria, resulting in increased overall survival rates. Therefore, that study provides strong evidence for an important contributory role for MCP-1 in the initiation of the renal inflammatory process. No reduction of circulating IC levels or renal Ig and complement deposits was observed for the MCP-1-knockout
Figure 7. Time course of renal proinflammatory cytokine mRNA expression. Twenty micrograms of total RNA isolated from whole kidney was analyzed using the mCK2b (A) and mCK3 (B) RPA kits from Pharmingen. Samples were organized in time groups, and each group was separated from the others by an empty lane. For each group, samples were loaded on the gel using the following scheme: first lane, MRL control sample (M); in the next three lanes, samples from three different MRL/lpr mice (ML). T, tRNA control sample (20 μg). (A) Increased expression of mRNA for interleukin-1β (IL-1β) and its corresponding regulator IL-1 receptor antagonist (IL-1Ra), as well as for interferon-γ inducible factor, in MRL/lpr mice, compared with MRL mice, starting at week 12. (B) Higher levels of lymphotoxin-β, tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and IFN-β expression in MRL/lpr mice, compared with the age-matched control animals. TGFβ, transforming growth factor-β; MIF, macrophage migration inhibitory factor.
mice, suggesting that activation of complement alone is not sufficient to promote leukocyte recruitment. This observation supports the generally accepted pathophysiologic mechanism of IC-mediated nephritis, in which MCP-1 is expressed secondarily to the deposition of IC and the activation of complement. Exposure of mesangial cells to IC has been demonstrated to stimulate the generation of chemokines such as MCP-1 and RANTES (40). As pointed out by Tesch et al. (19), the MCP-1 deficiency led to marked improvement of the glomerular pathologic features but to only partial reduction of the overall infiltration and especially the perivascular infiltration. This observation raises the issue of the potential role of other chemokines, in addition to MCP-1, in lupus nephritis. The recruitment of different leukocyte subpopulations is regulated by different chemokines, and our data provide clear evidence for this possibility, because multiple chemokines were upregulated.

In previous studies of human biopsies, predominantly CCR5-positive infiltrating mononuclear cells could be detected (21). Similarly, CCR5, CCR2, and CCR1 are upregulated in experimental IC nephritis in mice (41). Interestingly, CCR5 binds RANTES and MIP-1B, whereas MCP-1 binds to CCR2 and RANTES also binds to CCR1. All of these chemokines and chemokine receptors were upregulated in the MRL/lpr mice. Furthermore, IP-10 (a chemokine belonging to the CXC chemokine subfamily) was also upregulated. This is of interest because Romagnani et al. (42) reported that IP-10 causes proliferation of cultured mesangial cells. Furthermore, those authors demonstrated that mesangial cells express CXC chemokine receptor 3, the receptor for IP-10, and that CXC chemokine receptor 3 is upregulated in mesangial proliferative human IgA nephritis (42).

If chemokines are major factors contributing to the cellular infiltration, then their site of production should co-localize with the infiltrate. It has been reported that essentially all renal cells can be stimulated to produce certain types of chemokines in vitro (22–24). In the IC-mediated nephritis in MRL/lpr mice, we observed MCP-1 and RANTES expression located mainly within the glomerular mesangium and in subendothelial areas with the most prominent IC deposition. In vitro, IC have been demonstrated to stimulate chemokine expression by mesangial cells (40). In vivo, the administration of IgG Fe fragments prevents glomerular injury in IC nephritis by reduction of the expression of the chemokines MCP-1, IP-10, and CINC-1 (43). Together, these findings support a role for IC deposits in the initiation of chemokine expression.

Some peritubular and tubular staining for MCP-1 and RANTES localized to the brush border or tubular lumen was occasionally observed in immunohistochemical analyses. The luminal and brush border staining might be explained by nonspecific absorbance of urinary MCP-1 and RANTES. The fact that no tubular MCP-1 or RANTES expression was detected by in situ hybridization argues against chemokine production by tubular cells, at least at the time points examined.

Although there is overall concordance between our observations on MCP-1 expression in MRL/lpr mice and those described by Tesch et al. (19) for MRL/lpr mice and Zoja et al. (18) for NZB/W lupus mice, the latter authors also noted major MCP-1 expression in tubules, which we did not observe. The reasons for this discrepancy may be related to the age of the mice examined and differences in the methods and antibodies used. Furthermore, immunolocalization of chemokines does not necessarily indicate the site of production, because chemokines are soluble mediators that diffuse from the site of production and bind to the extracellular matrix. Chemokines can also be filtered (especially with proteinuria) and subsequently taken up by proximal tubular cells. On the basis of our results using in situ hybridization, tubular cells do not seem to contribute significantly to MCP-1 or RANTES production in MRL/lpr mice at the time point examined, i.e., 14 wk of age. Possibly, the degree of chronicity influences tubular chemokine production. Tesch et al. (19) observed tubular MCP-1 expression predominantly in 5-mo-old MRL/lpr mice, whereas our last time point was 16 wk of age. In fact, Zoja et al. (18) reported that renal expression in NZB/W mice was different at early and later time points. At 2 mo of age, mainly glomerular MCP-1 expression was detected, whereas tubular expression predominated in 6-mo-old mice. It has been proposed that proteinuria itself is able to induce tubular cells to express MCP-1 (44). Our findings of initial glomerular and subsequently interstitial chemokine production are consistent with a model in which glomerular injury initially causes glomerular chemokine production and infiltration, with the subsequent proteinuria leading to tubulointerstitial damage and hence to chemokine synthesis, resulting in turn in interstitial inflammatory cell infiltration and damage (45).

It has been proposed that, after chemokine production by local cells, the infiltrating leukocytes become a source of chemokine production, resulting in an amplification loop. This hypothesis would explain why mRNA expression continuously increased in the MRL/lpr mice. As observed using in situ hybridization, only a few cells in the prominent interstitial infiltrates expressed chemokines. Potentially, these few cells could be sufficient to cause an amplification loop.

Because MCP-1, RANTES, and MIP-1B were upregulated early in our lupus model, the subsequent upregulation of mRNA for CCR2 and CCR5 at week 12 and of mRNA for CCR1 by week 14 in the MRL/lpr mice is not unexpected. As indicated by data from human biopsies, the chemokine receptors CCR2 and CCR5 are present only on infiltrating leukocytes (21). The idea that this is also the case in mice is supported by the observation that increased levels of mRNA for CCR2 and CCR5 in lupus kidneys are apparent only at time points at which prominent cell infiltration can be observed. In addition, expression of both CCR2 and CCR5 mRNA could be detected by in situ hybridization only in the cell infiltrate. Within glomeruli, we cannot totally exclude the possibility of the localization of in situ signals to resident glomerular cells, e.g., mesangial cells. To date, however, we have not been able to localize chemokine receptors to mesangial cells in either human or murine systems. CCR2 was mainly localized to mononuclear cells that were CD3-negative, whereas CCR5 was primarily expressed on CD3-positive lymphocytes. In contrast, CCR1 upregulation did not parallel CCR2 and -5 upregu-
lation and leukocytic infiltration in the lupus model but occurred only at later time points (after 14 wk). Furthermore, as presented in Figure 5A, CCR1 is constitutively expressed in both MRL/lpr lupus mice and MRL control mice even at early time points. CCR1 can be expressed on cultured mesangial cells, and its expression can be stimulated by IFN-γ (22). The late upregulation of CCR1, after the enhancement of renal IFN-γ expression, would be consistent with these in vitro findings. The role of CCR1 in inflammatory diseases remains unclear. The late upregulation of CCR1 might be part of an anti-inflammatory response, an hypothesis that requires further testing. It is hoped that the future availability of antibodies will allow a cell type assignment for CCR1 in lupus nephritis.

After the initial IC insult and the early expression of chemokines, a leukocyte infiltrate and proteinuria appeared in MRL/lpr mice. Only at that time point did we observe upregulation of several proinflammatory cytokines, such as TNF-α, IFN-γ, IFN-β, interferon-γ inducible factor, and IL-1β (Figure 7). These proinflammatory cytokines have been demonstrated to participate in some of the pathophysiologic processes in lupus mice, such as leukocyte activation and induction of cell adhesion molecule expression. For example, IFN-γ has been demonstrated to be required for lupus nephritis; IFN-γ-deficient MRL/lpr mice were protected from lymphadenopathy and early death, and the severity of renal damage was markedly reduced (27, 46, 47). In kidney biopsies from patients with lupus nephritis, TNF-α and IL-6 mRNA expression was found to be elevated (48). In addition, the administration of anti-TNF-α antibodies was able to abrogate mercuric chloride-induced lupus-like autoimmune disease in rats (49). Because the expression of these proinflammatory cytokines is increased only in weeks 10 to 12, i.e., significantly later than the first increase in chemokine levels, these proinflammatory cytokines do not seem to play a role in the earlier events in disease initiation. Nevertheless, some differences in temporal expression patterns could be observed. For example, we observed that the pattern of TNF-α and IL-1β mRNA expression was sequential, in concordance with the observations by McHale et al. (26), who also described sequential expression of these two cytokines and related it to intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression in MRL/lpr mice. The infiltrating leukocytes may be the source of cytokine production, and the combination of glomerular leukocyte accumulation and cytokine production may contribute to the initiation of proteinuria.

Another observation that deserves comment is the equal expression of TGF-β1 and -β2 in kidneys from MRL control mice and MRL/lpr mice. TGF-β mRNA levels increased with age in both types of mice but did so equally. Elevated TGF-β levels have been implicated as a major factor responsible for glomerulosclerosis and renal fibrosis in various forms of renal disease, but TGF-β also acts as an anti-inflammatory cytokine (50). Because TGF-β is present in the matrix in an inactive form, our data on TGF-β mRNA levels cannot rule out a potential role of this cytokine in the progressive renal damage of murine lupus nephritis. In any case, our data on the equal TGF-β expression in control and lupus mouse kidneys raise the issue of the contribution of this cytokine in this form of renal disease.

In conclusion, our results strongly support the hypothesis that the expression of specific chemokines is an initiating event in experimental lupus nephritis. The time course analysis of chemokines during murine lupus nephritis suggests that they are early mediators required for the recruitment of mononuclear cells into the kidney. In addition, during the initiation and progression phases, chemokines are preferentially localized to the tissue compartments with IC deposition, i.e., the glomeruli and to some extent the interstitium. These are also the areas of subsequent mononuclear cell infiltration. Therefore, chemokines are early mediators of renal injury, and studies with antagonists for the respective chemokine receptors not only may provide a “proof of concept” but also may expand the limited repertoire of immunosuppressive drugs for the treatment of lupus nephritis.

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