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Most human nephritis is due to glomerular deposition and/or formation of immune complexes (IC). In cultured mesangial cells, Fc receptor stimulation induces proliferation, matrix synthesis, and release of several mediators implicated in the initiation and progression of glomerular injury. Since Ig Fc fragments in vitro modified these phenomena, we studied the effects of systemic administration of IgG Fc fragments on the evolution of experimental IC nephritis. Fc fragment injection (1 mg/day i.p.) to rats with ongoing nephritis (proteinuria 20–50 mg/24 h vs 9 ± 0.2 mg/24 h in controls) markedly ameliorates proteinuria, renal function, and morphological renal lesions. This was accompanied by a reduction in the renal synthesis of chemokines (monocyte chemoattractant protein-1, IFN-inducible protein-10, and cytokine-induced neutrophil chemoattractant-1), matrix proteins, and growth factors (platelet-derived growth factor, and TGF-β), and in the activity of transcription factors. The treatment did not affect the glomerular deposition of IgG IC and complement C1q. In contrast, a decrease in the renal expression and production of C3 was observed without changes in serum complement levels. In vitro, very low complement consumption and no C3b covalent interaction were observed with Fc fragments, confirming that they did not modify systemic complement activity. These results indicate that the administration of Fc fragments prevents the development of glomerular damage in an aggressive model of proliferative glomerulonephritis through mechanisms involving a reduced local generation of complement, chemokines and growth factors. Modulation of IC-mesangial cell interaction by Fc fragment administration could represent a new approach to the treatment of severe immune nephritis. The Journal of Immunology, 2000, 164: 2092–2101.
chemotactic proteins, adhesion molecules, and matrix proteins involved in inflammation, immunological responses, cell differentiation, and growth control (17, 19–22). NF-κB is a dimer of p50 and p65 subunits (19), and AP-1 is composed of homodimers and heterodimers of Jun and Fos proteins (20). The activation of these transcription factors during glomerular injury has been scarcely studied (23, 24).

In recent years, several groups have described the presence of FcR for IgG and IgA in rat and human MC (25–27) and several intracellular signals induced through FcR (28–30). The stimulation of resident MC with IC elicits synthesis of proinflammatory and profibrogenic cytokines and complement components, as well as mesangial proliferation and matrix production (18, 30–34). In vitro, these cell responses were markedly decreased by the presence of Fc fragments of Igs in the culture medium (18, 28, 30, 33). Therefore, we speculated that the in vivo administration of Fc fragments could attenuate the inflammatory response and other biological changes elicited by IC.

In a model of IC nephritis in rats, characterized by marked glomerular immune deposits, cell proliferation, matrix accumulation, and intense proteinuria (35, 36), we have studied the effect of systemic injection of highly purified Fc fragments of IgG in addition. We analyzed whether the therapeutic effect of Fc fragment administration may involve a reduced renal production of complement components (C3), chemokines (MCP-1, IP-10, and CINC-1), and growth factors (PDGF and TGF-β), as well as the attenuation of transcription factor activation (NF-κB and AP-1) that regulates their expression. The beneficial effect observed in animals treated with Fc fragments paralleled the effects observed in vitro, suggesting that FcR could be an important target in the therapeutic approach to severe immune nephritis. In addition, our data could be extended to other IC-mediated diseases.

Materials and Methods

Purification of IgG Fc fragments

Fc fragments from rabbit IgG were obtained by digestion with activated papain (Sigma, St. Louis, MO), which does not need reducing agents to assure the integrity of the inter-H-H disulfide bridge (37). The Fc fragment (molecular mass of about 60 kDa) was purified by affinity chromatography on a protein A-Sepharose column and gel filtration on a Superdex 75 column in PBS (Pharmacia Biotech, Uppsala, Sweden) and concentrated to 2 mg/ml, and did not contain any detectable Fab fragment, IgG, or other Igs. Purified Fc fragments and BSA (Sigma) were radiolabeled with 1 μCi Na125I (Amersham, Buckinghamshire, U.K.) using the chloramine-T method (sp. act., 0.1 mCi/mg). Fc fragments were biotinylated by incubation with sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce, Rockford, IL).

Experimental design

Preliminary studies were performed in healthy female Wistar rats to establish the optimal conditions for the Fc fragment treatment. Animals were injected i.p. with 1 mg of 125I-labeled Fc fragments, and at different time points the organ distribution and pharmacokinetics were determined in a gamma counter. As control, radiolabeled albumin was injected into a parallel group of animals to determine the nonspecific trapping of radioactivity. The specific distribution of Fc fragments was expressed as percentage of Fc fragments of Igs in the culture medium (18, 28–30). The synthesis of complement components (C3), chemokines (MCP-1, IP-10, and CINC-1), and growth factors (PDGF and TGF-β) was as follows: expression of C3b-IgG complexes was analyzed in frozen sections of C57Bl/6 mice treated with rabbit anti-rat IgG (Sigma), anti-rabbit IgG (Sigma), sheep anti-rat C3, rabbit anti-rat fibrinectin (36), and goat anti-rat type IV collagen (Southern Biotechnology, Birmingham, U.K.) Abs, respectively. Depositions of C1q were analyzed by immunoperoxidase with rabbit anti-human C1q Ab produced in our laboratory (40). The specificity of this Ab was demonstrated by Western blot using human and rat sera in comparison with purified human C1q. The quantification of infiltrating and proliferating cells was performed on frozen tissue specimens by an indirect immunoperoxidase technique with the following Abs:OX1 (pan-licokocyte CD45 Ag), OX19 (CD5 Ag), OX6 (rat MHC class II Ag, RT1-B), ED1 (monocyte/macrophages) (Serotec, Oxford, U.K.), and proliferating cell nuclear Ag (PCNA: Dako). Immunohistochemistry for chemokines was analyzed in paraffin-embedded renal tissues by incubation with goat anti-human MCP-1 (50 μg/ml; Immugenex, Los Angeles, CA), goat anti-mouse IP-10 (10 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), and goat anti-rat CINC-1 (10 μg/ml; R&D System, Oxon, U.K.). After treating with peroxidase-conjugated secondary Abs, the samples were developed with 3,3′-diaminobenzidine tetrahydrochloride and counterstained with hematoxylin. Negative controls were run in parallel with the omission of primary Abs.

Analysis of mRNA expression

Total RNA from rat renal cortex pieces was obtained by the acid guanidine-thiocyanate-phenol-chloroform method, and the mRNA expression of rat C3, TGF-β, and PDGF A chain was analyzed by RT-PCR using the following primers (Genosys, Cambridge, U.K.): C3 (693 bp): 5′ primer, 5′-GGA AGT GTG GTG AGG ATG GCA-3′; 3′ primer, 5′-CTG ATG AAT TGG GAA ACG ACC GCA-3′; TGF-β (298 bp): 5′-primer, 5′-CCG AGC TGC TCC ACA GAC GAG AAC TGC-3′; 3′ primer, 5′-CAC GAT CAT GTT GGA CAA CTC CTG C-3′; PDGF A chain (312 bp): 5′ primer, 5′-GAG ATG ACC CCG CGG GAT TTG AT-3′; 3′ primer, 5′-CTT CAC CCT CAC ACA TGG G-3′. A total of 1 μg of RNA was reverse transcribed to single-stranded cDNA, and then a PCR containing 20 pmol of primers, 0.5 μM [α-32P]dCTP (3000Ci/mmol; Amersham) and 3 U Taq DNA polymerase was conducted with annealing temperatures of 56°C (C3), 60°C (TGF-β), or 57°C (PDGF). The expression of G3PDH was used as internal control.
For in situ hybridization, riboprobes were generated by ligation of the PCR product into a PCR 3 vector (Invitrogen, San Diego, CA), and labeled anti-sense and sense cRNA probes were generated using T7 or SP6 polymerases and digoxigenin-labeled UTP as substrate (Boehringer Mannheim, Mannheim, Germany). Paraffin-embedded tissue sections were fixed in 1.5% paraformaldehyde-1.5% glutaraldehyde and treated with 5 mM levamisole, 0.2 N HCl, and 25 µg/ml proteinase K. Hybridization was conducted at 42°C with 0.4 µg/ml denatured digoxigenin-labeled riboprobes. Sections were washed and incubated with alkaline phosphatase conjugated anti-digoxigenin Ab (Boehringer Mannheim). Colorimetric detection of mRNA was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate. Negative controls consisted of matched serial sections hybridized without probe, with sense probe, or pretreated with 25 µg/ml RNase A.

Activity of transcription factors

Nuclear protein extraction from frozen renal tissue samples and EMSA were performed as described (24, 30). NF-κB and AP-1 consensus oligonucleotides were radiolabeled with [γ-32P]ATP (3000 Ci/mmol; Amer-sham). A total of 10 µg of nuclear protein was incubated in buffer containing 50 µg/ml poly(dI-dC) (Pharmacia), and 0.035 pmol of the labeled probe. The reactions were analyzed on a 4% nondenaturing polyacrylamide gel and autoradiographed. Specificity of the binding reaction was confirmed using a 100-fold excess of unlabeled specific oligonucleotides. Identification of nuclear proteins bound to the oligonucleotides was performed by using Abs against the p50, p65, and c-Rel subunits of NF-κB or the c-Jun and c-Fos subunits of AP-1 (Santa Cruz Biotechnology).

The Southwestern histochemistry was developed to detect the in situ distribution and DNA-binding activity of transcription factors (41). NF-κB and AP-1 consensus oligonucleotides were digoxigenin labeled with a 3’ terminal transferase (Boehringer Mannheim). Frozen and paraffin-embed- ded tissue sections were fixed in 0.5% paraformaldehyde and incubated with 0.1 mg/ml DNase I. The DNA-binding reaction was performed by incubation with 10 pmol of the labeled DNA probe in buffer containing 0.25% BSA and 0.5 µg/ml poly(dI-dC). The sections were then incubated with alkaline phosphatase-conjugated anti-digoxigenin Ab, and colorimetric detection was performed as described. Preparations without probe were used as negative controls, and mutant-labeled probe and excess of unla beled probe were used to test the specificity of the technique.

Evaluation of tissue staining and statistical analysis

In glomerular, tubular, and interstitial areas, around 20 fields from each animal were examined without any previous knowledge about the experimental design. The stainings in the different renal structures were graded semiquantitatively on a scale from 0 to 3 (0, negative; 1, weak; 2, moderate; and 3, strong staining), and data were expressed as means ± SEM. Statistical analysis was performed by ANOVA, Student-Newman-Keuls, or Tukey-Kramer tests. Differences were considered to be significant at p < 0.05.

Results

Tissue distribution of Fc fragments

Radiolabeled Fc fragments of IgG (1 mg) were i.p. injected into healthy control rats, and, after 3, 6, 9, and 24 h, animals were killed and the radioactivity in selected organs (spleen, heart, lung, liver, and kidney) was measured. The data of Fc fragment distribution were corrected for background, considered as the unspecific trapping of a non-Fc-related protein (radiolabeled albumin) injected into a parallel group of animals. The analysis of the specific distribution revealed that the maximal accumulation of Fc fragments was detected in liver and kidneys, peaking at 3 h after injection (17 and 18% vs injected dose, respectively) and decreased after 24 h (4 and 5%, respectively).

The biodistribution of Fc fragments in the kidney was analyzed in control rats injected with biotinylated Fc fragments. After staining with avidin-biotinylated peroxidase, positivity for biotin was found in the glomeruli, with a diffuse distribution, whereas no staining was observed in rats injected with unlabeled Fc fragments or PBS containing the biotinylation reactive (data not shown). Additionally, immunofluorescence studies with a specific Ab against rabbit IgG (no cross-reactivity with other species) showed the presence of Fc fragments in the glomeruli of control rats treated for 2 wk with 1 mg of Fc fragments (Fig. 1A). For all of these reasons, we thought it appropriate to perform the treatment with a daily i.p. injection of 1 mg of Fc fragments for 2 wk.

Proteinuria, renal function, and biochemical parameters

In the model of proliferative nephritis employed, proteinuria rises above normal values around the 9th wk after the first Ag injection (35, 36). When proteinuria reached 20–50 mg/24 h, animals were randomly assigned to receive either 1 mg/day of Fc fragments (Fc-treated group) or vehicle (untreated group) for 2 wk. At the start of therapy, proteinuria was 31 ± 6 mg/24 h, respectively (NS). As shown in Fig. 1, urinary protein excretion in untreated animals increased over the 2 wk of study, progressing to a full-blown nephrotic syndrome. The administration of Fc fragments prevented the development of intense proteinuria (86% re duction in relation to the untreated animals).

Table 1. Evolution of serum and renal parameters in experimental IC nephritis

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control + Fc Fragments</th>
<th>Untreated Nephritis</th>
<th>Nephritis + Fc Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteinuria (mg/24 h)</td>
<td>9 ± 0.2</td>
<td>7 ± 0.7b</td>
<td>592 ± 55c</td>
<td>81 ± 30d,e</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>69 ± 11</td>
<td>67 ± 15b</td>
<td>221 ± 27c</td>
<td>103 ± 16d,e</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.5 ± 0.1</td>
<td>3.4 ± 0.2b</td>
<td>1.9 ± 0.3c</td>
<td>3.1 ± 0.2d,e</td>
</tr>
<tr>
<td>Creatinine clearance (µl/min/100 g)</td>
<td>322 ± 2</td>
<td>319 ± 3b</td>
<td>203 ± 13b</td>
<td>300 ± 6c,d</td>
</tr>
<tr>
<td>Complement C3 (g vs standard rat serum)</td>
<td>103 ± 4</td>
<td>112 ± 9b</td>
<td>110 ± 6c</td>
<td>115 ± 6c,e</td>
</tr>
<tr>
<td>Complement CH50 (U/ml)</td>
<td>126 ± 5</td>
<td>105 ± 4b</td>
<td>103 ± 3b</td>
<td>120 ± 9c,e</td>
</tr>
</tbody>
</table>

a Serum and 24-h urine samples were collected at the end of the study, and the different parameters were measured by standard methods. The results are the means ± SEM of the individual values of animals from each group.

b,e Not significant and p < 0.05 vs healthy control rats, respectively.

d,e p < 0.05 and not significant vs untreated nephritic rats, respectively.
Untreated animals developed renal dysfunction, as evidenced by reduced creatinine clearance, hypoproteinemia, and hypercholesterolemia (Table I). Animals treated with Fc fragments showed a preservation of the creatinine clearance and near normalization of serum albumin and cholesterol levels. Healthy control rats receiving Fc fragments or vehicle displayed serum and renal parameters in the normal range (Table I).

Detection of immune deposits

This model is characterized by the presence of a large amount of immune deposits in the mesangium and glomerular capillary wall (36). Immunofluorescence analysis with anti-rat IgG revealed no significant differences in the deposition of IgG among glomeruli from untreated and Fc-treated rats (3 ± 0 vs 2.8 ± 0.1; semiquantitative score, NS) (Fig. 2, E and F). No staining for rat IgG was detected in control animals treated with vehicle (data not shown) or Fc fragments (Fig. 2D). The renal distribution of Fc fragments after injection was analyzed by immunofluorescence with anti-rabbit IgG. As indicated in Fig. 2, A and C, Fc fragment staining was similar both in control and nephritic animals treated with Fc fragments for 2 wk, whereas no staining was observed in nephritic rats injected with vehicle (Fig. 2B).

Immune deposits of several components of complement, such as C1q (Fig. 2K) and C3 (Fig. 2H), were present in the kidney of nephritic animals, mainly in the glomerular area and in some tubuli. The semiquantitative analysis revealed no significant differences in the localization and intensity of C1q between Fc-treated and untreated nephritic rats (2.5 ± 0.2 vs 2.7 ± 0.1, NS) (Fig. 2L). In contrast, a decrease in the glomerular staining of C3 was noted after Fc fragment administration (0.2 ± 0.1 vs 2.5 ± 0.3, p < 0.05) (Fig. 2L). Control rats injected for 2 wk with Fc fragments showed a very low staining for both complement components (Fig. 2, G and J).

Analysis of complement

Since recent data suggest that locally generated complement could be important in tissue injury (42–44), we studied the gene expression of C3 in renal tissues of rats. Fig. 3, A–C, is a representative example of in situ hybridization for C3 mRNA. Few glomerular and tubular cells positive for C3 mRNA were observed in the kidney of healthy control rats injected with Fc fragments (Fig. 3A) or vehicle (data not shown). In diseased kidney, C3 mRNA expression was increased in the glomerulus (with positivity found mainly in the mesangial area, and in some epithelial cells and infiltrating leukocytes. In contrast, a representative Fc-treated rat (C) presents only a slight mRNA expression of C3 (Fig. 3B).

FIGURE 2. Localization of injected Fc fragments and immune deposits. Immunofluorescence A–J and immunoperoxidase J–L analysis of the deposition of Fc fragments (A–C), IgG (D–F), C3 (G–I), and C1q (J–L). Representative micrographs from a healthy rat injected with Fc fragments as control (A, D, G, and J), untreated nephritic rat (B, E, H, and K), and rat with ongoing nephritis treated with Fc fragments (C, F, I, and L). The mesangial distribution of Fc fragments was similar in both control (A) and nephritic (C) animals receiving the treatment for 2 wk, whereas untreated nephritic rats (B) were negative. The immune deposits of IgG (E) and C1q (K) observed in nephritic rats were unmodified after the treatment (F and L). However, C3 staining in nephritic rats (H) was diminished by the treatment (I). Magnification, ×200 A–C and ×400 D–L.

FIGURE 3. Renal expression of C3 component of complement. In situ hybridization was performed with digoxigenin-labeled C3 anti-sense probe (A–C). Micrographs of a representative healthy control rat (A) with a small expression in some glomerular and tubular cells and a nephritic rat (B) showing increased expression of C3 in glomerular and tubular cells and infiltrating leukocytes. In contrast, a representative Fc-treated rat (C) presents only a slight mRNA expression of C3. Magnification, ×200. (D) The RNA from renal cortex of control (C), nephritic (N), or Fc-treated (Fc) rats was analyzed by RT-PCR (33 cycles) with specific primers for rat C3. The PCR products were electrophoresed, and densitometry of the bands was corrected by the G3PDH expression. Values of arbitrary densitometric units are means ± SEM of the individual animals from each group (control, n = 6; untreated nephritis, n = 9; and Fc-treated, n = 10) analyzed in triplicate (*, p < 0.05 vs control; **, p < 0.05 vs untreated nephritic rats).
The covalent interaction of serum C3 with IC was analyzed by SDS-PAGE experiments. The bands characterized those IC which have activated complement, bound C3b (band B), and are able to generate the C3/C5 convertase (band A). Representative of two different experiments.

**FIGURE 4.** Activation of the complement system by IC and Fc fragments. A. Hemolytic assay was performed with sensitized sheep erythrocytes incubated with control rat serum as complement source and increasing concentrations of IgG IC (▲) or Fc fragments (△). Consumption of complement was expressed as percentage of inhibition respect to hemolysis in control conditions (CH50 value of control rat serum). Results are means ± SEM of three different experiments performed in duplicate. B. The covalent interaction of serum C3 with IC was analyzed by SDS-PAGE after incubation of IgG IC (100 μg) in the absence (lane 1) or presence (lanes 2 and 3) of Fc fragments (100 μg). Two bands characterize those IC which have activated complement, bound C3b (band B), and are able to generate the C3/C5 convertase (band A). Representative of two different experiments.

Bowman’s capsule cells, tubular epithelial cells, and interstitial infiltrating cells (Fig. 3B). In contrast, the administration of Fc fragments diminished this local expression of C3, since a low amount of C3 transcripts was detected in rats with ongoing nephritis treated with Fc fragments for 2 wk (Fig. 3C; semiquantitative glomerular score, 0.4 ± 0.2 vs 2.8 ± 0.2, p < 0.01). The hybridization signal was highly specific, as assessed by several negative controls, such as hybridization with the sense probe or pretreatment with RNase A (data not shown). These results were confirmed by RT-PCR analysis (Fig. 3D). After densitometry and correction for the G3PDH expression, the 3-fold increase in the mRNA expression of C3 observed in the kidneys of rats with nephritis was reduced to control levels after Fc fragment administration (Fig. 3D).

The serum complement was analyzed by radial immunodiffusion for C3 and hemolytic assay. As shown in Table I, C3 and CH50 values were unmodified in healthy and nephritic animals injected for 2 wk with Fc fragments or vehicle, indicating that the treatment did not affect systemic complement. In addition, several in vitro experiments were performed to assess whether Fc fragment preparation could modify the activation of the complement system. Sensitized sheep erythrocytes were incubated with IC or Fc fragments in the presence of control rat serum as complement source. Hemolysis of sensitized erythrocytes (CH50 value) was dramatically decreased by incubation with increasing concentrations of IC, reaching a plateau of 100 μg/ml (Fig. 4A). In contrast, a very low consumption of complement was observed after incubation with Fc fragments (Fig. 4A). The maximal reduction of hemolysis was about 17% (300 μg/ml, Fig. 4A) even when very high concentrations of Fc fragments (0.5 and 1 mg/ml) were employed (data not shown). We also studied the effect of Fc fragments on the C3b binding to preformed IgG IC. Upon interaction of serum C3 with IC, C3b-C3b-IgG covalent complexes are immediately formed. These C3b-C3b dimers constitute the core for the assembly of C3/C5 convertase on the IC, which subsequently are converted to iC3b-iC3b-IgG by the complement regulators (38). These complexes were detected on SDS-PAGE and fluorography by two bands (of high molecular mass) with molecular composition C3a65-C3e43 (band A) and C3a65-H of the IgG (band B), which correspond to C3b-C3b and C3b-IgG covalent interaction, respectively, and that identify opsonized IC (37–39) (Fig. 4B, lane 1). The molecular mass observed for bands A and B in a highly cross-linked gel is different from that theoretically calculated, indicating the influence of the shape on the apparent molecular mass, as previously reported (39). In the presence of Fc fragments, the C3b covalent binding to IC was weakly enhanced (Fig. 4B, lanes 2 and 3). After densitometric analysis, we found that the intensity of bands A and B increased 128 ± 3 and 116 ± 4 (percentage vs control IC), respectively. However, we did not detect new bands corresponding to C3b-Fc covalent complexes. These results suggest that the Fc fragment was not sequestering C3.

**Table II. Inhibition of pathological changes in nephritis by Fc fragment administration**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Control + Fc Fragments</th>
<th>Untreated Nephritis</th>
<th>Nephritis + Fc Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total glomerular cell no.</td>
<td>48 ± 1.5</td>
<td>50 ± 2^b</td>
<td>130 ± 12^c</td>
<td>67 ± 9^b,d</td>
</tr>
<tr>
<td>Tubular lesions</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.1^b</td>
<td>2.6 ± 0.2^c</td>
<td>0.7 ± 0.3^b,d</td>
</tr>
<tr>
<td>Interstitial lesions</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>2.4 ± 0.4^c</td>
<td>0.3 ± 0.2^b,d</td>
</tr>
<tr>
<td>PCNA^c cells</td>
<td>1.2 ± 0.04</td>
<td>1.5 ± 0.1^b</td>
<td>29 ± 6^c</td>
<td>2.7 ± 0.4^b,d</td>
</tr>
<tr>
<td>Total leukocytes</td>
<td>1.0 ± 0.1</td>
<td>1.3 ± 0.1^b</td>
<td>23 ± 5^c</td>
<td>3.7 ± 0.5^b,d</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>0.25 ± 0.05</td>
<td>0.5 ± 0.1^b</td>
<td>6 ± 2^c</td>
<td>0.7 ± 0.1^b,d</td>
</tr>
<tr>
<td>ED1^d monocyte/macrophages</td>
<td>0.3 ± 0.01</td>
<td>1.0 ± 0.2^b</td>
<td>13 ± 3^c</td>
<td>3.0 ± 0.5^b,d</td>
</tr>
</tbody>
</table>

^a^ The morphological lesions in renal samples from the different groups of animals were studied after hematoxylin-eosin and Masson’s trichrome staining. Analysis of proliferating and infiltrating cells was performed by immunoperoxidase staining. Values represent cells/glomerulus or semiquantitative score (from 0 to 3) and are expressed as mean ± SEM. Statistical analyses are based on unpaired t test and ANOVA test.

^b,c^ Not significant and p < 0.01 compared to control rats, respectively.

^d^ p < 0.01 vs untreated nephritic rats.

**Glomerular cell proliferation and inflammatory infiltrate**

The morphological aspects of the glomerular lesions in this model have been described previously (35, 36). Quantification of renal damage is indicated in Table II. Animals with nephritis showed glomerular hypercellularity, mesangial expansion, severe necrotizing lesions, and inflammatory cell infiltration. In contrast, the Fc-treated animals showed a significant decrease in the structural glomerular damage, with discrete evidence of mesangial hypercellularity and matrix expansion. The tubulointerstitial lesions were also significantly reduced (Table II). Glomerular and tubular structures were well preserved in the kidney of control rats injected with Fc fragments or vehicle.

The glomerular hypercellularity of nephrititc rats could be due to the proliferation of resident cells and the recruitment of leukocytes. The glomerular proliferation was analyzed by staining with anti-
PCNA Ab, observing a marked increased in PCNA \(^{+}\) cells in glomeruli of untreated nephritic rats when compared with controls (Table II). In contrast, Fc fragment treatment was associated with a marked reduction in the number of proliferating cells. Fc-treated rats had also less glomerular infiltration of total leukocytes, T lymphocytes, and ED1\(^{+}\) monocyte/macrophages than rats with untreated nephritis (Table II).

Detection of chemokines in renal tissues

The presence of chemokines involved in the leukocyte recruitment was determined using an indirect immunoperoxidase technique. Control rats injected with Fc fragments or vehicle presented a low amount of MCP-1 in the mesangium, whereas staining for IP-10 and CINC-1 was negative (Fig. 5A, D, and G). Induction of the IC nephritis caused a marked increase in renal chemokine production at the end of the study. Untreated nephritic rats presented a strong staining for MCP-1, IP-10, and CINC-1 in glomerular and tubular cells, as well as in inflammatory cells infiltrating the interstitium.

FIGURE 5. Expression of MCP-1, IP-10, and CINC-1 proteins in renal tissue. Immunostaining was performed on renal tissues from healthy control rats (A, D, and G), untreated nephritic rats (B, E, and H), and Fc-treated rats (C, F, and I) using specific Abs against MCP-1 (A–C), IP-10 (D–F), and CINC-1 (G–I). The very low glomerular staining observed in control rats was dramatically increased after induction of IC nephritis. Treatment with Fc fragments largely prevented chemokine production. Magnification, ×200.

FIGURE 6. Immunolocalization of matrix proteins. Animals with nephritis showed positive staining for fibronectin (A) and type IV collagen (C) in the glomeruli. Rats injected with Fc fragments present a diminution in the glomerular deposits of fibronectin (B) and type IV collagen (D). Magnification, ×400.

FIGURE 7. In situ detection of growth factors in rats with IC nephritis. In situ hybridization of PDGF A chain (A–C) and TGF-\(\beta\) (D–F) from a control rat (A and D), untreated nephritic rat (B and E), or Fc-treated rat (C and F). In nephritic rats, PDGF A chain and TGF-\(\beta\) transcripts are present in mesangium and tubular cells. Treatment with Fc fragments attenuates the mRNA expression for both growth factors, observing a weak staining in glomeruli and some tubuli. Magnification, ×200.
The increase in chemokine production by both infiltrating and intrinsic kidney cells was largely prevented by Fc fragment treatment, with a slight positivity in glomeruli and some tubular cells (Fig. 5, C, F, and I). Semiquantitative evaluation revealed a significant decrease in the glomerular chemokine production in comparison with the untreated nephritic animals (1.5 ± 0.1 vs 3 ± 0.2, p < 0.01 for MCP-1; 0.5 ± 0.2 vs 2.5 ± 0.2, p < 0.001 for IP-10; 0.7 ± 0.2 vs 2.8 ± 0.1, p < 0.001 for CINC-1).

Immunolocalization of extracellular matrix proteins

In normal kidneys, fibronectin is present in the mesangium and along the glomerular basement membrane (data not shown). Immunofluorescence studies showed that fibronectin increased in the mesangium, capillary wall, tubular basement membrane, and interstitium of nephritic animals (Fig. 6A). Positive immunostaining for type IV collagen was seen along the glomerular basement membrane and in the mesangial matrix and tubules (Fig. 6C). Administration of Fc fragments diminished the glomerular deposition of both matrix components (1.6 ± 0.3 vs 2.9 ± 0.1, p < 0.05 for fibronectin; 1.2 ± 0.2 vs 2.8 ± 0.2, p < 0.01 for type IV collagen) (Fig. 6, B and D).

PDGF and TGF-β mRNA expression

The cellular distribution of these growth factors in the kidneys of animals from different groups was investigated by in situ hybridization. Kidneys from normal rats injected with Fc fragments as control expressed very low levels of PDGF and TGF-β mRNA (Fig. 7, A and D), which increased dramatically in animals with nephritis. Positivity for PDGF in nephritic animals was found mainly in the mesangium and in some tubular epithelial cells, particularly in distal tubules (Fig. 7B). Expression of TGF-β was seen in the mesangial area, the glomerular endothelial cells, and in infiltrating cells around the tubules (Fig. 7E). Animals treated with Fc fragments showed a minimal expression of both growth factors when compared with untreated rats (0.4 ± 0.2 vs 2.6 ± 0.2, p < 0.001 for PDGF; 0.5 ± 0.2 vs 2.7 ± 0.3, p < 0.01 for TGF-β) (Fig. 7, C and F). Hybridization with the sense probe was performed as negative control of the technique (data not shown). The in situ hybridization results were confirmed by RT-PCR analysis. As indicated in Fig. 8, the up-regulation of both PDGF and TGF-β mRNA expression observed in the kidneys of rats with nephritis (2- and 2.5-fold, respectively) was normalized to basal levels in Fc-treated animals.
Activated NF-κB with Abs against p50, p65, c-Jun, and c-Fos, suggesting that of the band intensity was observed after incubation of nuclear extract since the intensity of the bands was dramatically decreased (Fig. 9). The specificity of transcription factor activity was verified transcription factors was largely prevented by Fc fragment administration (Fig. 9). The activity of both transcription factors was largely prevented by Fc fragment treatment in both mesangial and tubular areas. Absence of nuclear labeling was detected in nephritic rat tissue after incubation with mutant NF-κB (D) or an excess of AP-1-unlabeled probe (H) used as negative controls. Magnification, ×400.

**FIGURE 10.** Localization of activated transcription factors. In situ detection of NF-κB (A–D) and AP-1 (E–H) was performed by Southwestern histochemistry using digoxigenin-labeled oligonucleotides in frozen and paraffin-embedded renal tissues, respectively. Representative glomeruli from a healthy rat injected with Fc fragments as control (A and E), and a nephritic rat showing positive staining in a large number of cells in the glomerulus and tubuli (B and F). The intense nuclear positivity for NF-κB (C) and AP-1 (G) was largely prevented by Fc fragment treatment in both mesangial and tubular areas. Absence of nuclear labeling was detected in nephritic rat tissue after incubation with mutant NF-κB (D) or with an excess of AP-1-unlabeled probe (H) used as negative controls. Magnification, ×400.

**Activation of transcription factors**

The corticomedullary transcription factor activity was analyzed in nuclear proteins by EMSA. Extracts from nephritic rats showed a marked increase in the intensity of the NF-κB and AP-1 bands, indicating transcription factor activation (Fig. 9). The activity of both transcription factors was largely prevented by Fc fragment administration since the intensity of the bands was dramatically decreased (Fig. 9). The specificity of transcription factor activity was verified by competition experiments with an excess of unlabeled probes. Moreover, the appearance of supershifted bands and/or diminution of the band intensity was observed after incubation of nuclear extracts with Abs against p50, p65, c-Jun, and c-Fos, suggesting that activated NF-κB and AP-1 complexes are constituted by p50/p65 and c-Jun/c-Fos proteins, respectively (Fig. 9).

The localization of the activated transcription factors in the kidneys was analyzed in situ by Southwestern histochemistry (41). Negative staining for NF-κB and AP-1 was observed in renal tissues from healthy control rats receiving Fc fragments (Fig. 10A and E) or vehicle (data not shown), whereas the number of positive cells was very increased in nephritic rats (Fig. 10, B and F). Transcription factors were widely distributed in glomerulus and tubulointerstitium, with positive nuclear staining in MC, glomerular epithelial cells, some tubular epithelial cells, and mononuclear infiltrating cells. In contrast, a small number of cells was stained in the group of rats treated with Fc fragments (Fig. 10, C and G) (semiquantitative score, 0.2 ± 0.1 vs 2.6 ± 0.1 for NF-κB; 0.2 ± 0.1 vs 2.5 ± 0.3 for AP-1). As negative controls to determine the specificity of the DNA-binding reaction, sections were incubated without the probe (data not shown), with the mutant-labeled probe (Fig. 10D), or with an excess of consensus unlabeled probe (Fig. 10H).

**Discussion**

Activation of cultured MC with IC triggers the release of inflammatory and fibrogenic mediators, cell proliferation, and matrix synthesis (18, 30–34). Some of these events were inhibited by the presence of Fc fragments of Igs in the culture medium, but not by F(ab′)2 fragments (18, 28, 30, 33), suggesting an important role of FcR in the pathogenesis of immune glomerular injury. Therefore, we hypothesized that the FcR stimulation in renal cells induced by IC could be interrupted in vivo by the Fc fragments administration. In a model of active IC nephritis in rats, we demonstrate that the exogenous administration of Fc fragments markedly ameliorates proteinuria, renal function, and glomerular and tubulointerstitial lesions. This was accompanied by a decrease in the renal expression of complement, chemokines, and growth factors, as well as in the activity of transcription factors.

The beneficial effects of Igs administration to patients with immune diseases have been extensively described, but the mechanisms are still not well defined (45–48). Among them, FcR blockade, anti-idiotypic Ab interactions, prevention of the complement activation, and modulation of immune function were considered (45). A number of studies has also reported that the administration of Abs against some chemokines, cytokines, and growth factors exerts a beneficial effect on the course of various experimental glomerulonephritis (9–11, 14, 16). However, given the complex and redundant cytokine network, it is relatively improbable that the pharmacological modulation of one single mediator could have a major role in the treatment of human glomerulonephritis. Since the interaction of deposited and/or locally formed IC with resident glomerular cells bearing FcR (MC and macrophages) is one of the first steps in the pathogenesis of immune renal injury (1), our therapeutic approach of Fc fragment injection could be more rational. In contrast to these published studies in which treatment with Igs or specific Abs was mostly prophylactic (9–11, 16, 45, 47), we administered the IgG Fc fragments when the animals had a certain kidney damage with a proteinuria two to five times higher than that of controls. This is the first report on the therapeutic approach of Fc fragment administration in experimental nephritis, but the results are in concordance with two recent studies performed in acute immune thrombocytopenic purpura (49) and xenogenic hyperacute rejection (50).

The Fc fragment preparations employed in this study were highly purified, and the tissue distribution clearly suggests that they were principally taken up by the kidney and liver and persisted for at least 24 h. The immunostaining revealed the presence of Fc fragments in the glomeruli of healthy rats several hours after injection. Additionally, the same pattern of mesangial localization, both in healthy and nephritic rats treated with Fc fragments, was observed at the end of the study, suggesting that the Fc fragment distribution into the kidney was not affected by the disease. The i.p. route was chosen because the IgG plasma levels in rodents employed higher doses of either IgG or Fc fragments (46–51). Thus, we consider that administration of Fc fragments could be more effective than intact IgG with additional advantages. The
lower m.w. of Fc fragments would allow a better localization in the injured kidney. In addition, Fc fragments contain all of the structural elements necessary to induce the effector functions of IgG. The Fc portion of IgG could also be easily obtained by gene technology avoiding the large pool of blood donors needed for the complete IgG molecule, thus providing a cheaper and safer treatment.

Since both deposited IgG IC and injected Fc fragments colocalize in the mesangium of Fc-treated nephritic animals, we postulate that the blockade of FcR could be the underlying mechanism of the beneficial effect of Fc fragments. As a consequence, activation of renal cells by IC and local production of inflammatory proteins involved in leukocyte recruitment could not occur. In this work, we show that the administration of Fc fragments markedly reduces the number of proliferating and infiltrating cells and the extracellular matrix synthesis in glomerular and tubulointerstitial areas. Among the molecules involved in these processes, chemokines and growth factors seem to play a major role. Chemokines are known to be secreted by infiltrating mononuclear and resident renal cells in response to various stimuli, including IC (30–32). The stimulation of cultured MC with IC via FcR also triggers the formation of extracellular matrix proteins, a process partially mediated by the autocrine synthesis of TGF-β (18). The increased expression of chemokines and growth factors was described in several models of renal injury (9–12, 15, 16, 24, 36), and the glomerular lesions were improved by administration of neutralizing Abs (9–11, 14, 16). In our experimental model, we observed that in temporal association with leukocyte infiltration and glomerular damage, there was an up-regulation of MCP-1, IP-10, and CINC-1, largely prevented by treatment with Fc fragments both at the glomerular and tubulointerstitial levels. Additionally, the renal localization of PDGF and TGF-β was analyzed by in situ hybridization, observing a reduced renal mRNA expression of both growth factors in parallel with a diminution in MC proliferation and extracellular matrix accumulation.

The Fc fragments might also attenuate IC-mediated renal injury through additional mechanisms. First, the Fc fragment preparation can decrease the formation, clearance, and deposition of IC into the glomerulus. This is unlikely because no changes in the localization and intensity of glomerular IgG were observed after the treatment. Second, the Fc fragments could have a systemic action on the serum complement levels. However, there was no evidence of systemic complement activation and depletion, since C3 and CH50 levels were unmodified by the treatment. These data are supported by the in vitro studies with Fc fragments, which revealed a very weak complement consumption. Third, Fc fragments can interfere in the kidney by preventing the local synthesis and/or activation of complement. Our finding of a decrease in the glomerular staining of C3 (an essential component of both classical and alternative pathways of complement) after Fc fragment injection supports this mechanism.

It is well known that very high doses of IgG inhibit the deposition of C3b onto target surfaces diverting nascent C3b from tissue-bound IC to fluid phase IgG, forming C3b-IgG covalent complexes (51). However, in our system, the Fc fragments themselves (at a similar concentration as IgG IC) were not acceptors of C3b, and we did not detect the presence of C3b-Fc covalent complexes. Interestingly, the increased renal C3 mRNA expression observed in nephritic animals was reversed to control levels by the treatment, suggesting that Fc fragments are blocking the renal synthesis of C3. The importance of the local C3 synthesis has recently been reported in C3-deficient mice (44). In the kidney, several authors have described an increased renal expression of complement components in cytokine-stimulated cultured renal cells (52) and in glomerulonephritis (42, 43), and proposed that the activated, locally synthesized C3, may change the size and/or charge barrier of the glomerular basement membrane, causing proteinuria (42). Moreover, IC enhanced the synthesis of C3 by cultured human MC via FcR (34). In a murine model of IC nephritis, a reduction of the disease was observed in mice with a genetic deficiency in the γ subunit of FcR, but not after decomplementation with cobra venom (5), indicating that complement activation induced by mesangial IC deposition contributes to the pathogenesis of immune renal injury but could not be sufficient to initiate the inflammatory response. Thus, our data suggest that one of the mechanisms of the beneficial effect of Fc fragments seems to be the inhibition of renal complement expression, without serum complement modification. This could be a potential advantage for their therapeutic use in humans.

Nuclear translocation of active transcription factors and binding to specific DNA sequences of responsive genes can lead to their enhanced transcription and subsequent protein synthesis (19, 20). Transcription factor activity has recently been found in experimental nephritis and in cultured MC stimulated with IC (23, 24, 29, 30). In this work, we analyze the activation and localization of NF-κB and AP-1, transcription factors involved in the gene regulation of complement components, chemokines, growth factors, and matrix components (17, 21–22). The intense nuclear staining observed in cells from glomeruli and tubulointerstitium of animals with untreated nephritis virtually disappeared in the Fc-treated group. Therefore, our data suggest that Fc fragments may prevent the stimulation via FcR and thus inhibit the intracellular signaling that leads to the activation of a variety of nuclear transcription factors. Consequently, the transcription of a large number of genes involved in inflammation and in tissue injury was prevented.

On the whole, we have demonstrated that the administration of Fc fragments to animals with IC nephritis prevents the appearance of severe nephrotic syndrome, renal failure, and glomerular and tubulointerstitial lesions by interfering with the initial steps of renal injury. These data suggest that the FcR could be an important target in the therapeutic approach to IC diseases. Prospective control trials are needed to assess whether Fc fragments administration, alone or in combination with immunosuppressive drugs, may play a beneficial role in the treatment of severe glomerulonephritis in humans.

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References


