

Prevention of Proteinuria by the Administration of Anti-interleukin 8 Antibody in Experimental Acute Immune Complex-induced Glomerulonephritis

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Summary

Glomerular infiltration by neutrophils is a hallmark of acute glomerulonephritis. The pathophysiological role of interleukin 8 (IL-8), a potent neutrophil chemotactic cytokine (chemokine), was explored in an animal model of acute immune complex-mediated glomerulonephritis by administering a neutralizing antibody against IL-8. Repeated injection of bovine serum albumin (BSA) into rabbits caused the deposition of immune complexes consisting of BSA and rabbit IgG in glomeruli. Histological analyses revealed a small but significant number of neutrophils in glomeruli and the fusion of epithelial cell foot processes. Concomitantly, urinary levels of protein and albumin increased markedly (3.20 ± 0.97 and 1.39 ± 0.53 mg/h, respectively) compared with those of untreated animals (0.77 ± 0.21 and 0.01 ± 0.01 mg/h, respectively). Anti-IL-8 antibody treatment decreased the number of neutrophils in glomeruli by 40% and dramatically prevented the fusion of epithelial cell foot process. Furthermore, treatment with anti-IL-8 antibody completely normalized the urinary levels of protein and albumin (0.89 ± 0.15 and 0.02 ± 0.01 mg/h, respectively). These results indicated that IL-8 participated in the impairment of renal functions in experimental acute immune complex-mediated glomerulonephritis through activating as well as recruiting neutrophils.

The acute phase of glomerulonephritis is characterized by glomerular infiltration by inflammatory cells and proliferation of mesangial cells in the glomerulus (1). Immunologically mediated processes have been proposed as the basis for acute glomerulonephritis (2, 3). Two types of immunological mechanisms have been documented to be involved in human glomerulonephritis as well as animal models: trapping of circulating immune complexes (2) and binding of circulating antibody to antigen on the surface of the glomeruli (3).

In several animal models of immune complex-induced glomerulonephritis, infiltration of leukocytes, particularly neutrophils, was observed as the earliest and predominant pathological change, although the precise mechanism and pathophysiological role of leukocyte infiltration has not been determined (2). We previously observed that the administration of a specific neutralizing antibody against a potent polypeptide neutrophil chemotactic cytokine, IL-8 (4, 5), inhibited neutrophil-dependent inflammatory reactions as well as neutrophil infiltration in several experimental animal models, including LPS-induced dermatitis (6) and lung reperfusion injury (7). Moreover, IL-8 is produced in vitro by mesangial

and endothelial cells in response to IL-1 or TNF- α (8). These results prompted us to speculate that IL-8 is involved in the leukocyte infiltration observed in the acute phase of immune complex-induced glomerulonephritis. Thus, we evaluated the role of IL-8 in experimental acute immune complex-induced glomerulonephritis in rabbits by administering a specific neutralizing anti-IL-8 antibody. We observed that anti-IL-8 antibody treatment prevented albuminuria as well as infiltration of neutrophils into glomeruli, inferring that IL-8 is also involved in neutrophil-dependent tissue injury to glomeruli initiated by deposition of immune complex.

Materials and Methods

Antibodies and Reagents. A mouse monoclonal antibody, WS-4 (IgG $_{1k}$ type), which can neutralize rabbit IL-8 as well as human IL-8, was prepared as previously described (5). This antibody does not crossreact with monocyte chemotactic and activating factor (MCAF), IL-1 α , β , TNF- α , IL-6, and granulocyte colony-stimulating factor (G-CSF) (5). A mouse monoclonal antibody against a component of *Toxoplasma gondii* (IgG $_{1k}$ type) was used

as a control antibody (7). Crystallized BSA was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). LPS from *Escherichia coli* 055:B55 was purchased from Difco Laboratories (Detroit, MI). CFA was obtained from Iatron (Tokyo, Japan).

Experimental Design. On day 0, male Japanese White rabbits weighing ~2 kg were injected subcutaneously with 8 mg of either BSA or mouse IgG, dissolved at 2 mg/ml in physiological saline, and mixed with an equal volume of CFA. On day 7, animals that were given BSA on day 0 received a single intravenous injection of 10 µg of LPS in combination with 500 mg of albumin dissolved in 2 ml of physiological saline. On day 8, rabbits were additionally given 10 µg of LPS in 1 ml of physiological saline intravenously. 4 h after the last administration of LPS, rabbits were killed to obtain renal tissue. Either anti-IL-8 (WS-4) or the control antibody (TpM-1) at a dose of 10 mg in 1 ml of physiological saline was administered intravenously on both day 7 and 8. Urines were collected using a metabolic cage at two time intervals; from day -1 to the time of injection of either BSA or mouse IgG on day 0 and from the injection time on day 7 to the time of killing.

Histopathological Studies. One portion of the renal tissue was fixed in 10% buffered formalin followed by embedding in paraffin and staining with hematoxylin and eosin as well as periodic acid-Schiff (PAS) reagent. The sections were evaluated by two independent observers without prior knowledge of the experimental design by light microscopy. The total number of PMN stained with an alkaline phosphatase substrate kit II (SK-5200) and infiltrating mononuclear cells were counted on 50 randomly chosen glomeruli. Another portion of renal tissue was frozen rapidly and stained with either FITC-conjugated anti-rabbit IgG or anti-BSA (Cappel Laboratories, Malvern, PA) as previously described (15). Renal tissue

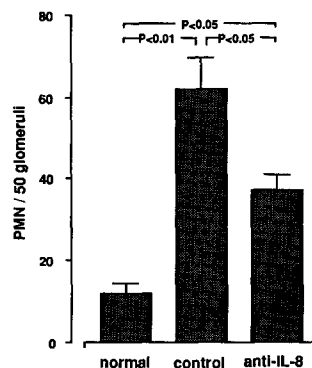


Figure 1. Effects of anti-IL-8 antibody on neutrophil infiltration into glomeruli. The numbers of neutrophils infiltrating glomeruli were determined in 50 randomly chosen glomeruli from each animal, examined by two independent examiners without prior knowledge about experimental procedures.

obtained from normal rabbits was used as a negative control. The amount and extent of fluorescence was evaluated in at least 50 glomeruli and was graded on an arbitrary scale from 0 to 3 (negative, scattered, weakly diffuse, and strongly diffuse). The degree of deposition was quantitated as an immune complex deposition index; the mean grade is shown. Several portions of each specimen were prefixed with 2.5% glutaraldehyde and postfixed with 4% osmic acid, progressively dehydrated in an alcohol series, embedded in Epok 812 (Nagase Industries, Tokyo, Japan), and cut into ultrathin sections. These were double-stained with uranyl acetate and lead citrate and were examined by electron microscopy (model H-600; Hitachi, Tokyo, Japan). Finally, the presence of the IL-8 molecule was immunohistologically examined on the ethanol-fixed renal tissue specimens by the indirect avidin-biotinylated peroxidase complex method using a specific murine monoclonal anti-human IL-8 (clone WS-4) antibody (5) and a peroxidase kit



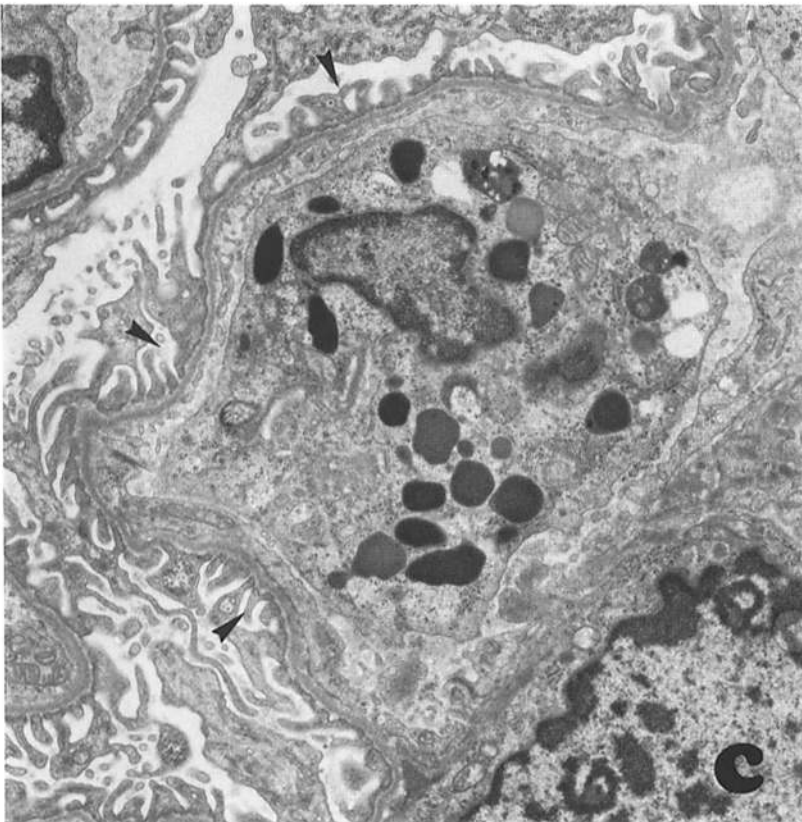


Figure 2. Representative electron micrographs of glomeruli showing fusion of the epithelial foot processes. There was no fusion of epithelial foot processes of the free wall of glomerular capillaries in normal rabbits (*a*). Glomeruli from rabbits treated with control antibody exhibited a high incidence of fusion of epithelial cell foot processes (*arrowheads*), especially in the vicinity of infiltrating leukocytes (*b*, original magnification $\times 3,000$). In contrast, no fusion of epithelial foot processes occurred in rabbits treated with anti-IL-8 antibody (*c*, original magnification $\times 3,000$), even in the presence of infiltrating leukocytes in glomerular capillaries (*arrowheads*).

(Vectastain ABC; Vector Laboratories, Inc., Burlingame, CA). Murine IgG was used as a negative control antibody.

Urinary and Plasma IL-8 Measurements. Plasma was collected at killing and urines were kept at -70°C until the measurement of IL-8. Both urinary and plasma IL-8 levels were determined by ELISA, as previously reported (6, 7). All assays were performed at least in duplicate. The detection limit of this ELISA system for IL-8 was <50 pg/ml. Urinary IL-8 was standardized by the excreted amount of urinary creatinine.

Determination of Urinary Protein and Albumin Concentration. Urinary protein concentrations were determined by the pyrogalol red method (10), whereas urinary albumin was measured by turbidimetric assay (11) using rabbit serum albumin as standard. Urinary protein and albumin excretion was expressed as the total amount excreted in 1 h (mg/h).

Statistical Analysis. The mean and standard error were calculated on all the parameters determined in this study. Statistical analyses were performed using paired and unpaired Student's *t* test for parametric data and Wilcoxon single-rank sum test for non parametric data. *p* <0.05 was accepted as statistically significant.

Results and Discussion

Histopathological Studies. Immunofluorescence analysis revealed no deposition of BSA and rabbit IgG in glomeruli from three untreated rabbits (data not shown). Granular deposits of both BSA and rabbit IgG were observed along the glomerular capillaries of 9 rabbits treated with BSA plus the control antibody and 10 rabbits treated with BSA plus anti-IL-8 antibody. Semiquantitative evaluation of deposition revealed no significant difference in the deposition of either BSA or rabbit IgG between glomeruli from rabbits treated with anti-IL-8 and rabbits given control antibody (data not shown), indicating that IL-8 had no effect on the process of immune complex deposition. Infiltration of 62.7 ± 7.6 PMN/50 glomeruli was observed in renal tissue from nine rabbits treated with BSA plus the control antibody (Fig. 1). The administration of anti-IL-8 antibody decreased the number of infiltrating PMN by $>40\%$ ($37.7 \pm 3.7/50$ glomeruli) although the number did not return to the level seen in renal tissue from normal rabbits (Fig. 1). These results indicate that IL-8 was at least partially responsible for the PMN infiltration observed in immune complex-induced glomerulonephritis. Since the anti-IL-8 antibody is highly potent, inhibition of the binding of rabbit IL-8 to rabbit neutrophils by 50% is obtained at a molar ratio of antibody/IL-8 of 1:1 (6), it is very unlikely that the dose of anti-IL-8 monoclonal antibody was insufficient to neutralize IL-8. Thus, these results suggest simultaneous generation of additional chemotactic factor(s), such as C5a, a potent leukocyte chemotactic factor (12), leukotriene B₄ or other arachidonic acid-derived lipophilic chemotactic factor(s) (13) or other chemokines, in particular, gro's (14). The latter possibility is supported by the fact that inflammatory stimuli including LPS, IL-1, and TNF- α induce various types of cells to produce gro's (14) as well as IL-8.

The number of mononuclear cells/50 glomeruli of three normal rabbits was 13.0 ± 1.8 . There was no difference be-

tween the number of mononuclear cells present/50 glomeruli in 9 rabbits given the control antibody (59.7 ± 24.5) and 10 rabbits treated with anti-IL-8 antibody (45.4 ± 22.6). These results do not exclude the possibility that other types of leukocytes, such as lymphocytes and monocytes, might contribute to glomerular injury over longer periods. This possibility will be examined by histological examination of renal tissue obtained from a different experimental model of glomerulonephritis in the chronic phase.

Electron microscopical analyses demonstrated few fusion of epithelial foot processes in the free wall of the glomerular capillaries from three normal rabbits (Fig. 2 a). In contrast, epithelial cell foot processes of all tufts were fused in all tufts, especially if there were infiltrating leukocytes nearby, as examined in at least 10 glomeruli from each of 9 rabbits injected repeatedly with BSA in conjunction with the control antibody (Fig. 2 b). In 10 rabbits treated with anti-IL-8 antibody, little fusion of epithelial foot processes occurred, even though leukocytes accumulated in glomerular capillaries (Fig. 2 c).

Urinary and Plasma IL-8. 19 normal untreated rabbits excreted only trace amounts of IL-8 into the urine (2.6 ± 2.6 pg/d). However, urinary IL-8 increased significantly at day 8 in nine rabbits that were repeatedly injected with BSA plus the control antibody (80.2 ± 38.4 pg/d), whereas plasma IL-8 was not detected in any rabbits. Next, we examined immunohistochemically the presence of antigenic IL-8 in renal tissue. In the glomeruli from three normal rabbits, IL-8 was not detected immunohistologically (data not shown). IL-8-positive cells, probably infiltrating mononuclear cells and mesangial cells, were observed in almost all glomeruli (at least 50 glomeruli from each specimen were examined) from nine rabbits that were repeatedly injected with BSA (Fig. 3 a). The staining was specific for IL-8, as control murine IgG did not result in positive staining (Fig. 3 b). These results favor the notion that IL-8 was locally produced in the glomeruli, and that IL-8 was causally involved in tissue injury in acute immune complex-induced glomerulonephritis by inducing infiltration of leukocytes, particularly neutrophils. The precise identification of the cellular sources of IL-8 remains to be done. Resident cells, such as mesangial cells and endothelial cells as well as infiltrating cells are possible sources of IL-8, based on the capacity of these cells to produce IL-8 in vitro (4, 8).

Effects of Anti-IL-8 Antibody on the Urinary Excretion of Protein and Albumin. 19 normal, untreated rabbits excreted only minute amounts of protein as well as trace amounts of albumin in the urine (0.77 ± 0.21 , 0.01 ± 0.01 mg/h, respectively) (Fig. 4, a and b). In contrast, all of the nine rabbits treated with BSA plus the control antibody excreted markedly elevated amounts of both protein and albumin in the urine (3.20 ± 0.97 , 1.39 ± 0.53 mg/h, respectively) (Fig. 4, a and b). The administration of anti-IL-8 antibody dramatically decreased the excreted amount of protein (0.89 ± 0.15 mg/h) and albumin (0.02 ± 0.01 mg/h) (Fig. 4, a and b). This indicated that anti-IL-8 antibody normalized the excretion of both protein and albumin, but not the neutrophil

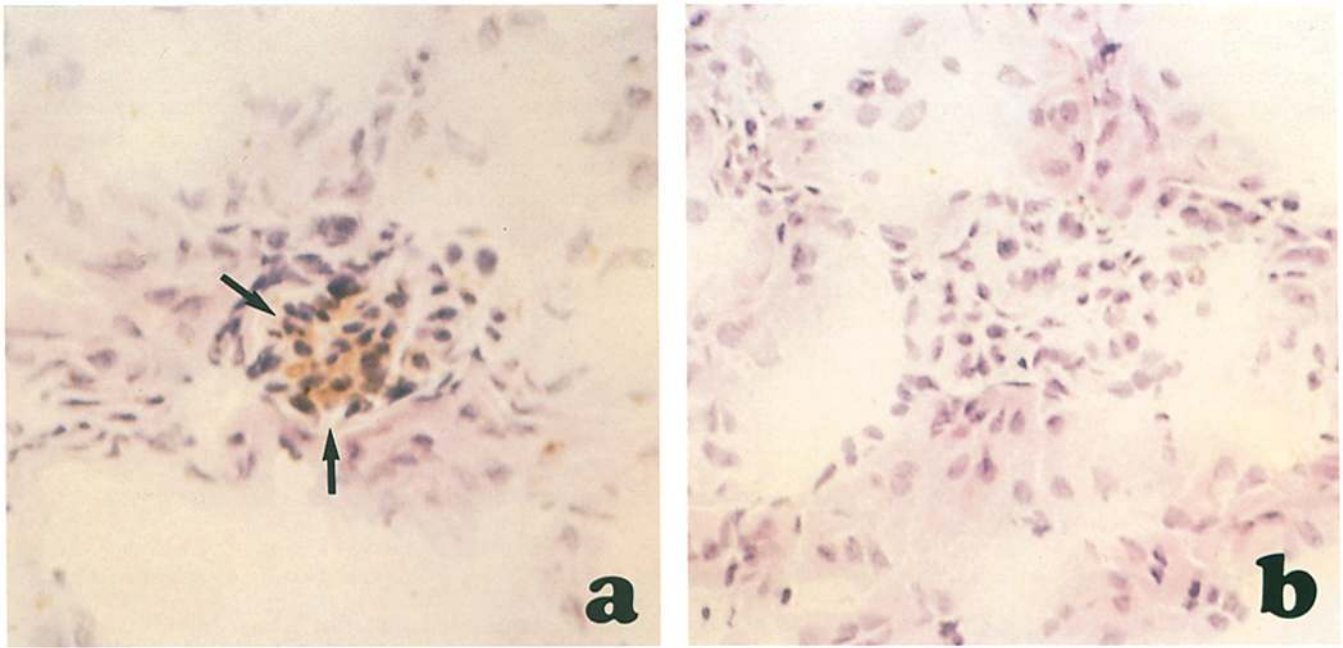


Figure 3. Immunohistochemical examination of glomeruli for expression of IL-8 protein in renal tissue. This could be detected using specific monoclonal anti-human IL-8 antibody at $\times 250$ original magnification (*a*, arrows). A representative result from 10 rabbits treated with control antibody is shown. Positive staining was not observed using control murine IgG on the same rabbit at $\times 250$ original magnification (*b*).

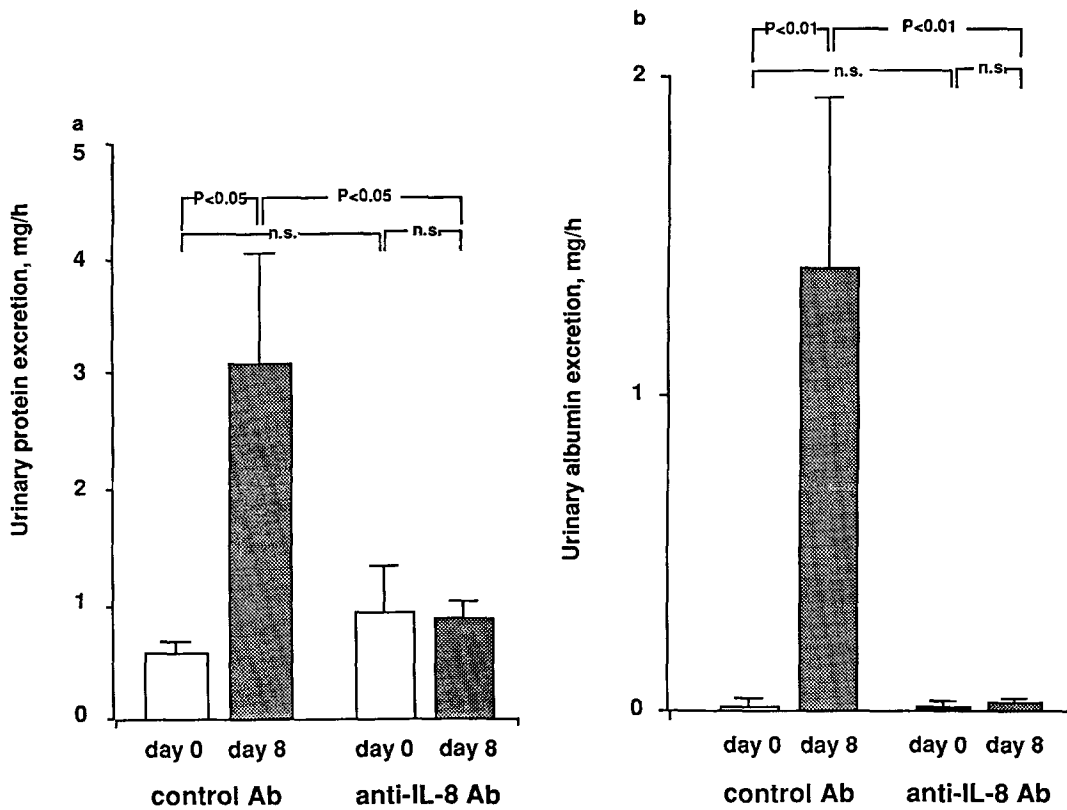


Figure 4. Effects of anti-IL-8 on urinary protein excretion (*a*) and urinary albumin excretion (*b*). Results from 9 rabbits treated with control antibody and 10 rabbits treated with anti-IL-8 antibody are shown. n.s., not significant.

infiltration. Electron microscopical analysis demonstrated that anti-IL-8 antibody treatment prevented the fusion of epithelial foot processes even though neutrophils were still infiltrating into the glomerular capillaries, inferring that anti-IL-8 antibody treatment blocked impairment of glomerular function almost completely.

The involvement of lysosomal enzymes, nitrous oxide (NO), and reactive oxygen intermediates (ROI) from leukocytes has been reported to play an essential role in inducing tissue injury in glomerulonephritis (15, 16). IL-8 has been documented to induce the release of lysosomal enzymes (14)

and generation of superoxide anions (14) from neutrophils in addition to being a chemoattractant for neutrophils. Thus, it is tempting to speculate that IL-8 is causally involved in the induction of glomerular injury by recruiting activated neutrophils that might be the source of lysosomal enzymes, NO or ROI, and that treatment with anti-IL-8 monoclonal antibody inhibits the activation as well as the infiltration by neutrophils. Collectively, administration of an antagonist to IL-8 could provide a key to future treatment of the acute phase of human glomerulonephritis.

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