

Distinctive Roles of Neutrophils and Monocytes in Anti-Thy-1 Nephritis[#]

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SUMMARY

Anti-Thy-1.1 glomerulonephritis as an experimental model for mesangial proliferative glomerulonephritis was induced in Wistar rats by a single injection of monoclonal IgG2a-anti-Thy-1.1 antibody (ER4G). This transient model is complement-mediated and leads to mesangial-cell (MC) lysis followed by MC proliferation, glomerular microaneurysm formation, glomerular influx of polymorphonuclear leukocytes (PMNs) and macrophages, proteinuria, and hematuria. In this study we investigated the distinctive roles of infiltrating PMNs or monocytes/macrophages by treating rats with an antibody against rat integrin CD11b/CD18 (ED7) or by depletion of monocytes with multilamellar clodronate liposomes, respectively. ED7 administration resulted in reduction of the influx of PMNs in glomeruli during the first 6 days after induction of Thy-1.1 nephritis, whereas treatment with an isotype-matched irrelevant antibody (PEN9) or with phosphate-buffered

saline had no effect on macrophage influx. Increased glomerular C3 and C6 deposition on days 1 and 3 was seen in the ED7-treated rats but not seen in the control groups. In addition, the ED7-treated group showed an increased number of aneurysmatic glomeruli and more severe hematuria. Monocyte/macrophage depletion led to a significant reduction of mesangial matrix expansion, although mesangial proliferation, proteinuria, and hematuria remained unaltered. These results, together with the known effects of PMN-derived enzymes on C3 cleavage, suggest that a reduction in the influx of PMNs results in sparing of C3 and consequently of more complement activation in the glomerulus with increased complement-mediated damage. Our data indicate that infiltrating PMNs and monocytes/macrophages play distinctive roles during inflammation in this model of MC glomerulonephritis.

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INTRODUCTION

The contribution of polymorphonuclear leukocytes (PMNs) and macrophages in glomerular injury has been well established. The influx of neutrophils during anti-thymus nephritis is well documented (1), but its role in the pathogenesis is largely unexplored(2). Injection of antibodies against Thy-1.1, a transmembrane glycoprotein on mesangial cells (3,4), results in complement-dependent mesangial-cell lysis (1,5,6), apoptosis (7), and subsequent mesangial proliferation (8) and extracellular-matrix expansion(9). Previous studies have shown that mesangial-cell injury and the sub-sequent proliferative response depend on complement activation and that the response can be suppressed by complementation with cobra venom factor (2,10). Similar results were demonstrated by Bagchus et al (11) who found no glomerular lesions after the injection of a non-complement-fixing monoclonal anti-Thy-1 antibody. During the development of mesangioproliferative glomerulonephritis, the glomeruli are infiltrated by neutrophils (1,2,12) and monocytes (13) Glomerular infiltration by neutrophils is characteristic of acute experimental glomerulonephritis (14,15) Proteinuria resulting from neutrophil-mediated glomerular injury has been shown in nephrotoxic serum nephritis (9,16) and crescentic glomerulonephritis (17) and after intrarenal injection of phorbol myristate acetate (PMA),(18) or of cobra venom factor, which causes complement activation and subsequent PMN chemotaxis (12,19) In each of these described models, PMN depletion markedly diminished proteinuria and resulted in reduction or attenuation of renal disease (17) The β 2 -integrin CD11b/CD18 has been shown to be involved in the influx of PMNs and monocytes/macrophages in different reperfusion models in rabbit (20) and experimental allergic encephalomyelitis (EAE) and acute colitis in rats (21,22). In the latter models, a mouse monoclonal antibody (mAb) against CD11b (ED7) was able to reduce the influx of inflammatory cells (predominantly PMNs) into the inflamed tissues and also the severity of the induced disease. In EAE, no effect of administration of anti-CD11b antibodies ED7 or ED8 was observed on the cellular infiltration (predominantly monocytes), but clinical severity was significantly reduced in the anti CD11b-treated EAE rats (21). The involvement of monocytes and macrophages in various glomerulonephritides has been clearly established (23). In particular their involvement has been associated with proliferative forms of glomerulopathies (24,25). Experimental evidence has been provided by rodent models of glomerulonephritis (26–28) However, in some cases infiltrating monocytes may serve merely to remove immune complexes and not to contribute to the glomerular injury (29) Recent interest has focused on the role of macrophages in the pathogenesis of focal glomerulo-sclerosis (30,31) Both

glomerular hypercellularity and expansion of the extracellular matrix are thought to be of primary importance in the development of capillary obsolescence and glomerulosclerosis. In the remnant kidney model in the rat, Van Goor and colleagues (23) showed that macrophages play a central role in the development of focal glomerulosclerosis. As described by Floege et al, the development of glomerulosclerosis in this model is preceded by mesangial proliferation and mesangial matrix expansion (32). In this study we investigated the role of glomerular monocytes and PMNs in the development of mesangial injury and subsequent glomerular hypercellularity and mesangial matrix expansion. The PMN infiltration into the glomeruli during Thy-1 nephritis was affected by pretreatment with monoclonal antibody ED7. Monocyte depletion was performed using a macrophage suicide technique, by injecting liposomes, in which clodronate was encapsulated (33). This study shows that each of these inflammatory cells plays a distinctive role in the pathogenesis of mesangioproliferative glomerulonephritis.

MATERIALS AND METHODS

Animals

Female inbred Wistar rats (160–185 g) were obtained from the animal facilities of the Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands. The rats were housed in accredited animal facilities and fed pelleted food, and they had access to water *ad libitum*. Experiments were performed in accord with Dutch legislation for the care and use of laboratory animals.

Antibodies

mAb against Thy-1.1 (mouse IgG2a/k against rat CD90) was derived from hybridoma ER4G as previously described (11). It was purified from ascitic fluid by affinity purification on protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden). Rabbit anti-mouse IgG was obtained from Jansen (Beerse, Belgium), and goat anti-rat fibrinogen-fluorescein isothiocyanate (FITC) was from Nordic (Tilburg, The Netherlands). The ED1 antibody is a murine monoclonal IgG1 to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (34). PC10 (Dako, Glostrup, Denmark) is a murine immunoglobulin M (IgM) mAb against proliferating cell nuclear antigen (PCNA), which is expressed by actively proliferating mesangial cells (35). Mouse mAb directed against C6 was kindly provided by Dr. W. G. Couser (Division of Nephrology, University of Washington, Medical Center, Seattle, WA). FITC-conjugated rabbit anti-rat C3 antibodies were generated in our own laboratory. Rat mAb anti-mouse IgG1-horseradish

peroxidase (HRP) was from Sanbio (Uden, The Netherlands). The accumulation of platelets was detected by mAb PI-1 (33). The glomerular influx of PMNs was assessed with FITC-labeled mAb W3/13. After the original description of its tissue distribution (36) and purification (37), the antigen recognized by mAb W3/13 has been identified as leukosialin (38), which is present on T cells, neutrophils, and brain from the rat. After molecular cloning of leukosialin, it has been designated as CD43. The expression and function of CD43 on neutrophil granulocytes have been described in several studies (39,40). It is well established that, during the course of anti-Thy-1 nephritis, an influx of neutrophils and monocytes/macrophages takes place (1). However, there has been no T cell infiltration, during this type of experimental mesangial cell-proliferative glomerulonephritis, that has been detectable with mAb R73 against the T cell receptor, although CD43-positive PMNs were observed with mAb W3/13. Therefore, our study and several earlier ones chose mAb W3/13 for immunohistochemical detection of PMNs in kidney sections of rats (1). ED7 is an mAb against cell adhesion molecule CD11b/CD18 and was prepared as described earlier (41). PEN9 is specific for penicillin and was used as an isotype control for ED7. PEN9 has been characterized (13).

Preparation of Liposomes

Multilamellar liposomes were prepared as described earlier (33). In brief, 86 mg phosphatidylcholine (a gift from Lipoid KG, Ludwigshafen, Germany) and 8 mg cholesterol, molar ratio 6:1, were dissolved in 20 ml methanol/ chloroform (1:1) in a round-bottomed flask. The thin film that formed on the interior of the flask after low-vacuum rotary evaporation at 37°C was dispersed in 10 ml phosphate-buffered saline (PBS; 10 mmol/L, pH 7.4), containing either 2.5 g dichloromethylene diphosphonate (Cl2 MDP; a gift from Boehringer Mannheim GmbH, Mannheim, Germany) or PBS, by gentle rotation for 10 min. Free Cl2 MDP was removed by rinsing the liposomes with PBS and centrifuging them for 30 min at 100,000 *g* at 16°C. The liposomes were then resuspended in 4 ml of PBS; 2 ml were intravenously injected per rat. Controls received 2 ml of PBS-encapsulated liposomes. The efficacy of depletion was evaluated by immunohistology with ED1 on kidney biopsies taken after 24 hours. Pilot experiments had shown that administration of higher concentrations of Cl2 MDP-liposomes led to a profound complement depletion, resulting in a decrease of proteinuria development and subsequent reduction of anti-Thy-1–induced pathology (data not shown). Therefore a liposome dose was chosen that did not affect the CH50 levels in the blood.

Induction of Anti-Thy-1 Glomerulonephritis

Mesangial proliferative glomerulonephritis was induced by injection of mAb against Thy1.1 (ER4G) at a dose of 1 mg/kg intravenously in the rat-tail vein. The animals were housed in metabolic cages, and 24-hour urine protein excretion was measured daily, using the biuret standard method. Urine containing 0.1% merthiolate was used.

Hematuria was assessed with dipsticks (Hema-Combisticks; Bayer Diagnostics, Mijdrecht, The Netherlands), which have been shown to be as sensitive as direct microscopic enumeration of erythrocytes in full urine. Moreover, the used dipstick can be examined to distinguish between small differences in the amounts of erythrocyturia. Because the values are comparable with those obtained by a colorimetric method and a direct counting of erythrocytes, we used the values obtained from dipsticks (1).

Experimental Design

For ED7-treatment, 21 young female Wistar rats were divided into three groups of 7 rats each. To determine the role of PMNs during anti-Thy-1.1 nephritis, one group of rats was injected, 2 hours before and 3 days after injection of ER4G, with 0.5 mg of mAb ED7. As controls, we used two groups of seven rats each, injected with PBS alone or with an isotype-matched irrelevant antibody, PEN9 (0.5 mg dissolved in equal amounts of PBS as for ED7). All antibodies were administered by tail vein injection. From all rats, renal biopsies were taken 1, 3, 6, and 10 days after injection of ER4G. Urine samples (24 hours) were collected before the experiment and on days 1, 2, 4, 6, 8, and 10.

For macrophage depletion studies, 54 female Wistar rats were used. One group of rats ($n = 24$) was injected with 1 ml/100 g of body weight liposome-encapsulated CI2 MDP at day 21 and with 1 mg/kg mAb ER4G at day 0. Kidney biopsies ($n = 8$ at each time point) were performed at 1, 4, and 24 hours after ER4G injection. At days 2, 6, and 14, eight animals were sacrificed. Corresponding groups of rats served as controls; six animals received liposome-encapsulated CI2 MDP followed by saline injection; in nine animals, only ER4G was injected at day 0; eight animals received liposome-encapsulated PBS followed by ER4G on day 0; four animals received liposome-encapsulated PBS followed by saline injection; six animals received saline only. Blood samples were collected from the tail vein before the injection of CI2 MDP and immediately before the administration of ER4G to evaluate CH50 levels. All intravenous injections, blood samples, and biopsies were performed under ether anesthesia. At sacrifice, the

kidneys were perfused *in situ* with PBS. Cortical tissue was processed for light and immunofluorescence micro-copy.

All experiments were carried out in two distinct sets. One complete set of experiments is presented in this study.

Histological Examination

Kidney tissue was obtained for light and immunofluorescence microscopy. For light microscopy, tissues were fixed in methacarn solution, dehydrated in graded ethanols, and embedded in paraffin. Sections (4- mm each) were stained with periodic acid/Schiff (PAS) reagent. The degrees of glomerular mesangiolysis (glomerular aneurysms) 6 and glomerular extracellular matrix expansion were graded semiquantitatively in 25 representative glomerular cross sections per rat, as described previously by Floege et al (42). Mean values per biopsy were calculated for the number of proliferating (PCNA⁺) cells and monocytes/macrophages per glomerular cross section. An indirect immunoperoxidase method was used for the identification of monocytes with mAb ED1 and for PCNA with PC10. Specific antibody binding was revealed by the reaction with hydrogen peroxide and diaminobenzidine (Sigma Chemical Co., St. Louis, MO). Normal rat spleen served for ED1 as positive control. Immunofluorescence microscopy was performed on tissue samples snap-frozen in CO₂–ice-cooled isopentane and stored at 270°C. Cryostat sections of 3 mm each were obtained and, after air-drying, fixed in acetone for 10 minutes at room temperature. The slides were washed twice in PBS and examined for the presence of rat C3 by a directly FITC-conjugated antibody. For the detection of PMNs and C6, tissue sections were preincubated with PBS containing a 1:300 dilution of a 30% H₂ O₂ solution (Merck, Darmstadt, Germany), to block endogenous peroxidases. Thereafter the slides were washed and incubated with digoxigenin (DIG)-conjugated mouse monoclonal anti-rat C6 or the DIG-conjugated mAb W3/13 (1) in 0.5% Boehringer blocking reagent (TNB) after incubation with HRP-conjugated Sheep F(ab') anti-DIG fragments. The slides were subsequently incubated with tyramide-FITC (NEN-Dupont Research Products, Boston, MA) for 30 minutes at room temperature (1). Mean values per biopsy were calculated for the number of PMNs per glomerular cross-section. Glomerular C3, C6, fibronectin, and IgG2a depositions were scored by a semiquantitative method as previously described (42), with scores ranging from 0 to 4 (0 = 0–5%, 1 = 6–25%, 2 = 26–50%, 3 = 51–75%, 4 > 75% of the glomerular cross-section). The slides were coded, and 20 glomerular cross-sections per rat were scored independently for each parameter by two experienced microscopists.

Photographs were taken on Kodak TX-400 film on a Leitz microscope equipped with a 4-mm BG 38 1 5-mm BG 12 filter for FITC.

Statistical Analysis

Mean values ($\pm$ SD) for each parameter were calculated and compared in an independent Student's *t*-test. A *P* value smaller than 0.05 was considered significant.

RESULTS

Anti-CD11b Treatment

Glomerular binding of mouse mAb ER4G showed a mesangial pattern with no differences between the ED7- treated and control groups. The antibody was detectable in the rat glomerulus until day 3. The influx of PMNs into the glomerular cross-section as assessed by detection with W3/13 was maximal on day 1. The PBS- and the PEN9-treated groups revealed 2.0 ± 0.2 and 2.1 ± 0.2 PMNs per glomerular cross-section, respectively. The number of PMNs detected in the ED7-treated group was significantly reduced to 1.6 ± 0.1 PMNs per glomerular cross-section ($P < 0.05$). A comparable reduction of PMNs was measured on days 3 and 6 of the experiment with highly significant values (Figures 1 and 2). Equal results of glomerular PMN counts were obtained by using PAS-stained sections.

The influx of monocytes/macrophages into the glomerular area did not reveal any differences between the various groups. In all rats, we observed a peak influx of this cell type at day 1, followed by a subsequent decline to baseline levels at day 10 (data not shown).

The glomerular deposition of C3 and C6 was scored semiquantitatively after indirect immunohistochemical staining of frozen kidney sections of each rat. Significantly higher scores for C3 and C6 deposition were found on day 1 for the rats treated with ED7 as compared with the PBS- and the PEN9-injected rats. C6 deposition was seen only in rats treated with ED7 on day 3. At later time points, no C6 deposition was detected in any of the experimental animals. Glomerular C3 deposition was highest on day 1 in the ED7-treated group and significantly increased as compared with the controls. On day 6, only ED7-treated rats exhibited some C3 deposition (Figures 1 and 2).

When the biopsies of the rats were analyzed by light microscopy, we observed a significantly higher maximum of glomerular microaneurysms ($37.1 \pm 16\%$) on day 3 for the rats receiving ED7. The glomerular lesions of the control

groups were approximately 50% less compared with ED7-treated animals (Figures 2 and 3). The extracellular matrix/fibronectin score was not significantly different

between the ED7-treated and the control groups. Also, the number of proliferating glomerular cells was not changed after reduction of glomerular influx of PMNs.

Figure 1

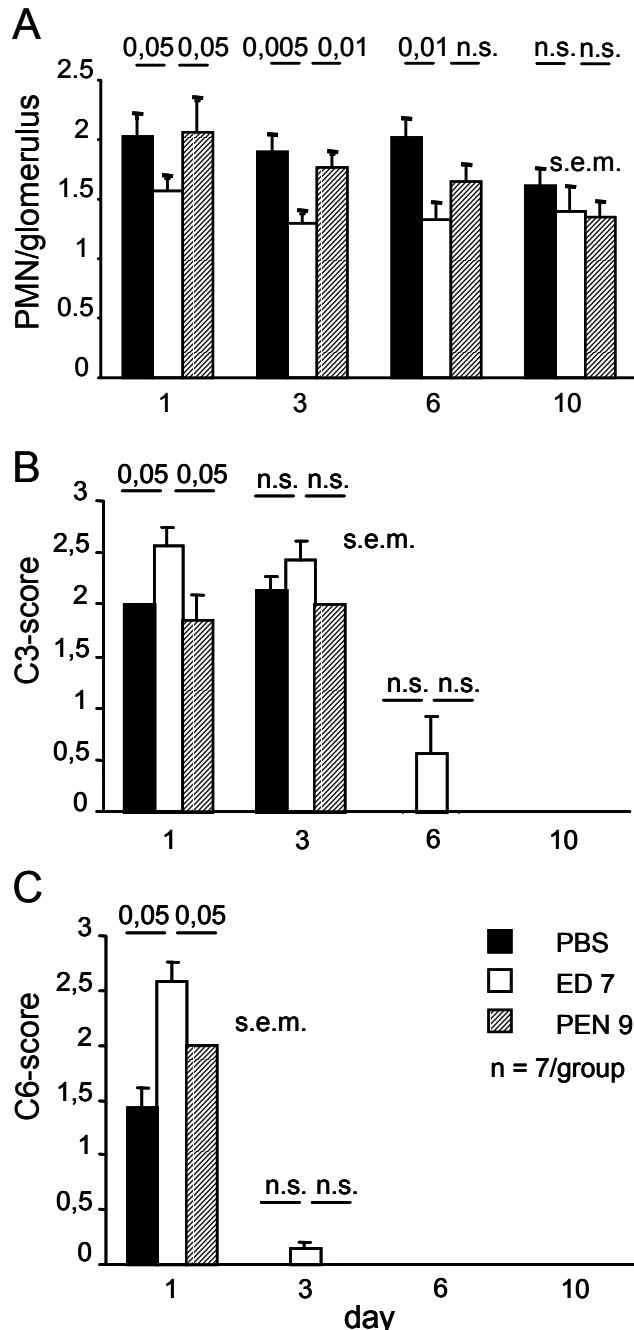


Figure 1. A: PMN influx in ED7-treated (white bars), PEN9-treated (pat-terned bars), and PBS-treated (black bars) rats. Immunohistochemical staining for intraglomerular PMNs is shown as mean $\pm$ SEM number of W3/13-positive cells/glomerular cross section per group. ED7 pretreatment led to significant reduction of PMN influx during anti-Thy-1.1 nephritis until day 6. At day 10, no significant difference was detected. **B:** Immunohisto-chemical staining for glomerular C3 deposition after induction of anti-Thy-1.1 nephritis, using a semiquantitative scoring system (shown as mean $\pm$ SEM). Higher scores for C3 were observed on days 1 and 3 for the ED7-pretreated group as compared with the control groups. Only the ED7-pretreated rats showed residual C3 deposition on day 6. **C:** Immunohistochemical detection for glomerular C6 deposition was found to be higher on day 1 in the ED7 group (shown as mean $\pm$ SEM). On day 3 only little C6 deposition was still found in this group, whereas the control groups did not show positivity for C6.

Hematuria and proteinuria

Hematuria from day 4 and later in this experiment showed, for the ED7-treated rats, higher values as compared with the control groups. Because of the high

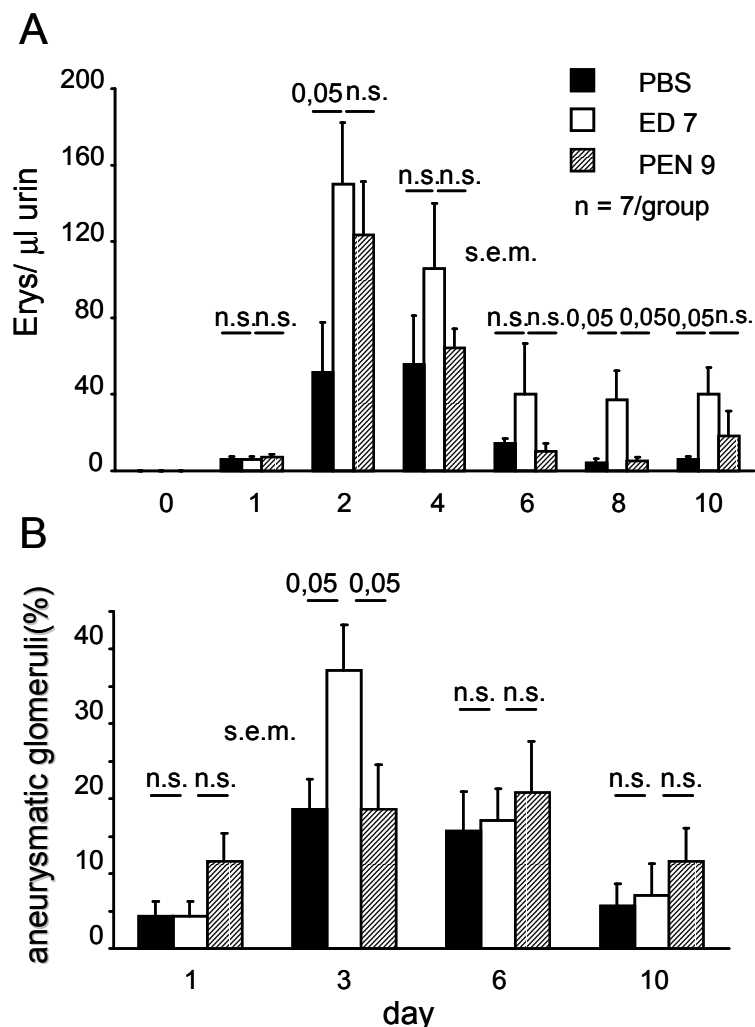
standard deviations in all groups, this increase was significant only on days 2, 8, and 10 (Figure 3). For proteinuria we did not find significant differences between the groups (data not shown).

Monocyte and Macrophage Depletion Studies

Rats treated with clodronate liposomes exhibited a nearly complete reduction of glomerular monocyte infiltration. Although control rats showed about 4.2 ± 1.8 cells per glomerulus, the monocyte-depleted rats had 0.2 ± 0.25 ED1⁺ cells ($P < 0.001$; Figure 4). A reduction of $15 \pm 23\%$ of circulating monocytes was found. It is interesting that no additional effect of ED7 was seen on the clodronate-mediated reduction in circulating monocytes.

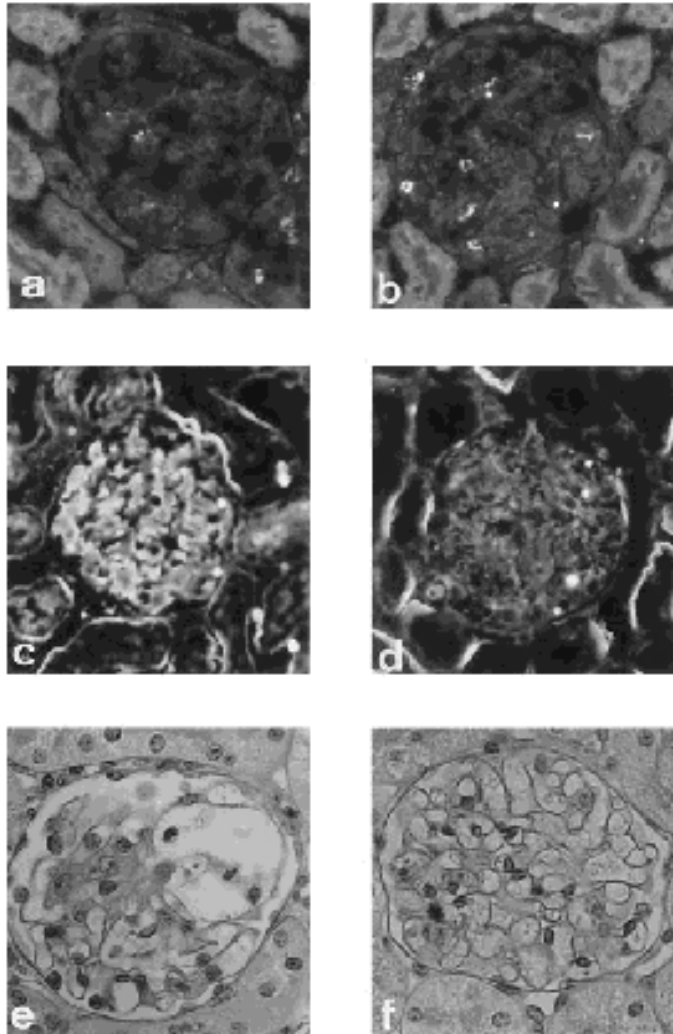
Figure 2. A: Course of hematuria during the experiment detected by dip stick. At all time points, hematuria of the ED7-treated group was highest compared with the control groups. Because of the high standard deviations, only the differences on days 2, 8, and 10 were significant. The data show mean values per group $\pm$ SEM. **B:** Detection of glomerular microaneurysms by scoring PAS-stained kidney sections (mean values per group $\pm$ SEM). The ED7-pretreated group developed a significantly higher percentage of glomeruli with microaneurysms on day 3 after induction of nephritis. The aneurysms detected on the other days of the experiment were not significantly different.

Figure 2



The influx of PMNs into the glomeruli was not affected by the monocyte/macrophage depletion. Glomerular deposition of C3 and C6 was not changed at all by macrophage depletion. All groups showed the expected degree of complement deposition as described for the control groups in the ED-7 experiment.

Figure 3. Immunohistochemical staining for PMN influx in ED7- and PEN9-/PBS-treated rats was performed using mAb W3/13. **A:** ED7 treatment led to significant reduction of PMN influx during anti-Thy-1.1 nephritis. A representative glomerulus obtained on day 1 is shown. **B:** The control groups showed significantly higher values of W3/13-positive cells. **C and D:** Immunohistochemical staining for glomerular C3 deposition on day 1 after induction of anti-Thy-1.1 nephritis are shown. **D:** Rats treated with control antibody PEN9 or PBS showed less deposition of C3 as compared with rats treated with ED7 (**C**). **E and F:** Glomerular microaneurysms were scored in PAS-stained kidney sections. The ED7-treated group of rats developed significantly more glomerular microaneurysms (**E**) on day 3 as compared with rats treated with PBS or PEN9 (**F**).



In the PAS-staining, we observed no differences for glomerular micro-aneurysms /mesangiolytic. However, a significant difference in extracellular matrix scores was found between the two groups (Figure 4). The glomerular deposition of fibronectin corresponded to the difference in mesangial extracellular matrix expansion (data not shown).

We did not observe significant differences in proliferating (PCNA⁺) mesangial cells between the different groups of rats (data not shown).

Proteinuria and Hematuria

In monocyte-depleted Wistar rats, proteinuria and hematuria were comparable with that in the control rats (data not shown).

DISCUSSION

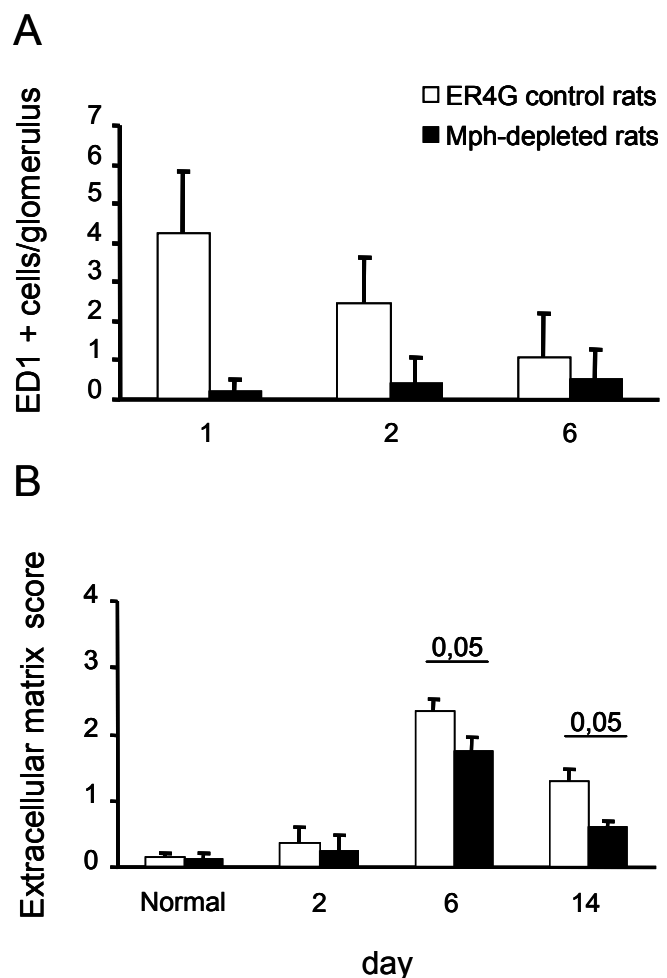
In this study we demonstrate that treatment of rats with ED-7, a mouse anti-CD11b mAb, selectively reduces glomerular PMN influx during the course of anti-Thy-1.1 nephritis. PMN reduction was associated with enhanced hematuria, a higher percentage of glomeruli with micro-aneurysms, enhanced glomerular deposition of C3 and C6, and no significant change in proteinuria, suggesting that microaneurysm formation is an important factor in determining the degree of hematuria.

Figure 4. A: Macrophage influx in macrophage-depleted (black bars) and control (white bars) rats. Immunohistochemical staining for intraglomerular macrophages is shown as mean number of ED1-positive cells per glomerulus per group $\pm$ SEM. Macrophage depletion led to an almost complete reduction of infiltrating macrophages on days 1 and 2 after induction of anti-Thy-1.1 nephritis. At day 6, no significant difference was detected. **B:** Extracellular matrix expansion was scored using PAS staining of rat kidneys. During anti-Thy-1.1 nephritis, glomerular extracellular matrix expansion (shown as mean $\pm$ SEM) was significantly reduced at days 6 and 14 after depletion of macrophages as compared with rats not depleted (white bars).

It is known that PMNs contribute to tissue injury during ischemic reperfusion of the pulmonary, coronary, cerebral, and splanchnic circulation. Injection of antibodies against CD18

confers protection against ischemic-reperfusion injury of these organs in many experimental models (43). Previously it was shown that ED7 is able to reduce the influx of neutrophils during experimental acute colitis (22) in rat. Also the damage in the colon was found to be much less. In a peritoneal recruitment assay, ED7 treatment reduced the influx of myelomonocytic cells (21). Also, in a lung

Figure 4



reperfusion model, an anti-CD18 antibody prevented vascular injury associated with a reduction of myeloperoxidase in the examined tissues (20,44).

No effect of ED7 treatment on monocyte influx during anti-Thy-1.1 nephritis was observed. The reason for the different behaviors of the two cell types in this model is unknown. A possible explanation could be the major pathogenetic role of complement in this model, in which several chemoattractants are released during the initial phase of injury, which may contribute to the attraction of monocytes.

Earlier experiments assessed the influence of mAb against CD18, CD11a, CD11b, very late antigen-4 (VLA-4), intercellular adhesion molecule-1 (ICAM-1), and E-selectin on glomerular neutrophil accumulation and proteinuria in Long-Evans rats with nephrotoxic-serum nephritis (45). This model is characterized by up-regulation of vascular cell adhesion molecule-1 (VCAM-1), ICAM-1, and E-selectin expression, rapid neutrophil infiltration into the glomerulus, and proteinuria. Treatment of animals with antibodies against CD18, CD11b, and ICAM-1 caused 63%, 46%, and 54% reduction, respectively, in proteinuria and 79%, 66%, and 54% reduction, respectively, in glomerular neutrophil counts, suggesting an important role for these adhesion molecules in this model of experimental nephritis (45). Moreover, these data indicate that neutrophils play a major pathogenetic role in experimental nephrotoxic nephritis, specifically in glomerular permselectivity, whereas other factors, for example, complement activation, seem to have a secondary role. These findings are further supported by another study investigating nephrotoxic-serum nephritis (46).

Our own results for the reduction of glomerular PMN counts are in agreement with the above mentioned studies. On the other hand, we did not find a reduction of hematuria or proteinuria in this complement-mediated experimental glomerulonephritis, suggesting that neutrophils do not significantly contribute to loss of permselectivity in this model. This is in agreement with earlier studies. (2,6,10).

In contrast, PMNs seem to contribute to the repair process during anti-Thy-1.1 nephritis. Maybe proteolytic enzymes released by these cells have a complement-clearing function, and consequently a reduction in PMN influx may be responsible for the observed pronounced and sustained C3 and C6 deposition in rats treated with ED7, compared with the controls. In the literature a complement-clearing function of proteases released from PMNs has been described. It was found that proteolytic enzymes derived from PMNs in pleural empyema or from isolated PMNs can inactivate C3 bound to Sepharose (47). Furthermore, cleavage of C3b and C3bi bound to human erythrocytes was shown to depend on purified

leukocyte enzymes or crude extracts from human PMNs (48,49), indicating a complement-clearing function of PMN-related enzymes. Because complement is the major pathogenic factor leading to MC lysis in this experimental model, this could be an explanation, on one hand, for the increased damage and hematuria observed in this study. On the other hand, it was found (50) that incubation of highly purified human C1 inhibitor with equally pure human leucocyte proteinase 3 resulted in a dose- and time-dependent inactivation of C1 inhibitor hemolytic activity, which may lead to an increase of activated complement factors.

The major difference in the above mentioned nephrotoxic nephritis is that, during Thy-1 nephritis, neutrophils and complement together play a role, whereas, in nephrotoxic nephritis, neutrophils seem to be the main factor affecting the permselectivity of the damaged rat kidneys. Neutrophils in nephrotoxic nephritis seem to contribute to mesangial injury, whereas neutrophils during Thy-1 nephritis do not seem to play a major role.

Concerning the role of macrophages, we found that selective depletion of macrophages led to a reduced glomerular matrix expansion. In contrast to ED7 treatment, glomerular complement deposition, microaneurysm formation, and hematuria remained unaffected.

These data are in agreement with recently published investigations (51). It was found that, after induction of anti-Thy- 1.1 nephritis, influx of macrophages is strain dependent. Lewis rats showed high amounts of infiltrating macrophages into the rat glomerulus, whereas F344 rats showed no enhancement of these infiltrating cells. Moreover, after transplantation of F344 kidneys in Lewis rats and vice versa, there was influx of macrophages only in kidneys transplanted to Lewis rats and not in kidneys transplanted to F344 rats. In F344 rats, the influx of macrophages was low, and the extracellular matrix expansion was marginal. In contrast, high amounts of infiltrating macrophages led to a highly significant increase of extracellular matrix in Lewis rats (51). The mechanisms leading to these differences are still unclear; all grafts showed an equal induction of monocyte chemotactic protein-1 in both rat strains. These data indicate a predominant factor located outside the kidney that leads to mononuclear infiltrates in this experimental disease. The potential mechanisms by which macrophages mediate glomerular-cell proliferation and the development of mesangial matrix expansion could involve release of cytokines by the macrophage itself or could involve stimulation by the macrophage of other cell types to release cytokines within the glomerulus (52,53). During tissue repair processes, macrophages can release TGF- β 1 (54), a growth factor that has clearly been shown to be involved in mesangial matrix expansion (54–56). A second macrophage-derived product with a possible high impact is nitric oxide, which is

abundantly secreted by infiltrating monocytes in this model (57). Monocytes have been shown to induce increased transcription of TGF- β and fibronectin (58).

In coculture studies, Mosquera demonstrated that monocyte-derived culture supernatants could induce mesangial cells to synthesize fibronectin *in vitro* (59). As shown in Figure 3, the mesangial expansion in monocyte-depleted rats was not completely inhibited. This may be because 1) especially major histocompatibility complex-II neg resident macrophages are less sensitive to liposome-mediated elimination (E De Heer, V Cattell, unpublished results), and 2) cytokines from other sources, for instance platelet-derived growth factor, may be able to induce mesangial matrix expansion, albeit less effectively (52).

In conclusion our study presents evidence that monocytes are specifically involved in the expansion of the mesangial extracellular matrix, whereas other immunopathological processes in the mesangium (complement activation, platelet aggregation, and mesangial proliferation) remain unaffected. These findings indicate that the mesangial alterations occur through distinct signaling pathways.

Taken together we hypothesize that a reduced influx of PMNs and a subsequent reduction in generation of C3-cleaving enzymes from PMNs may lead to a relative sparing of activated C3, which, on its own, results in more injury (glomerular aneurysms) and up-regulation of hematuria via a complement-dependent mechanism. Furthermore, we found monocytes being selectively involved in mesangial extracellular matrix expansion, whereas the induction of mesangial proliferation is induced through a separate pathway.

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