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An Evaluation of the Neutrophil as a Mediator of *In Vivo* Renal Ischemic-Reperfusion Injury

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Previous studies indicated that administration of a monoclonal antibody (MoAb 60.3), which blocks neutrophil adherence to rabbit endothelial cells, prevents ischemic-reperfusion (I-R) injury in multiple extrarenal organs. These findings indicated that the neutrophil can be a critical mediator of I-R tissue damage. To assess whether the neutrophil affects renal I-R injury, MoAb 60.3 was given to rabbits that were then subjected to either 50 minutes or 38 minutes of renal ischemia induced by renal artery occlusion (RAO). The severity of kidney damage was assessed 24 and 48 hours later by blood urea nitrogen and plasma creatinine concentrations and by renal histology. The results were compared with those obtained in time-matched RAO controls. MoAb 60.3 conferred no functional or morphologic protection against either severe or mild ischemic insults. To further evaluate whether neutrophils affect renal I-R injury, rats were depleted of neutrophils (<200 cells/mm^3) by anti-neutrophil serum administration and then they were subjected to either 37 minutes or 29 minutes of RAO. Neutrophil depletion conferred neither functional nor morphologic protection when compared with time-matched RAO controls. It was concluded that the uniform lack of protection noted in these experiments, despite that two different animals, two different ways of interfering with neutrophil function, and differing severities of ischemic injury were studied, strongly suggests that the neutrophil is not a critical participant in the renal I-R injury process. (Am J Pathol 1989, 135:509–515)

In recent years there have been many suggestions that reactive oxygen species (ROS) help mediate ischemic-reperfusion (I-R) injury in virtually every organ system, including the kidney.[1-5] Because investigation of these highly reactive compounds is made difficult by their short half lives and extremely low concentrations, which complicate their detection, their importance has been inferred by demonstrations of increased tissue products of lipid peroxidation during reperfusion and by showing that antioxidants confer protection.

Two principal mechanisms have been proposed for the generation of ROS in postischemic tissues. First, ischemic conversion of xanthine dehydrogenase to xanthine oxidase, in addition to the generation of hypoxanthine from ATP during oxygen deprivation, is believed to drive ROS formation during reperfusion.[6] The importance of this mechanism within the postischemic kidney has been a subject of recent debate.[5,7] Second, ROS may be generated by circulating neutrophils that can adhere to injured endothelium.[8] The potential relevance of this latter pathway to renal I-R injury has recently been suggested in a study by Linas et al.[9] These workers demonstrated that infusion of human neutrophils into postischemic isolated perfused rat kidneys augmented reperfusion injury, compared with kidneys perfused with albumin alone. That this neutrophil-induced exacerbation of injury could be blocked by simultaneous catalase infusion and that neutrophils from patients with chronic granulomatous disease (which lack the ability to generate H_2O_2) had no effect on the reperfusion process strongly suggest that ROS were responsible for the neutrophil-induced exacerbation.

One way of assessing whether or not neutrophils help mediate tissue injury *in vivo* is to assess whether neutrophil depletion (eg, by antineutrophil serum [ANS] or nitrogen mustard administration) confers protection. Three recent studies used this approach to assess whether the neutrophil plays a role in *in vivo* ischemic acute renal failure (ARF). Hellberg et al[10] reported that neutrophil deple-

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tion with ANS caused a modest increase in the immediate reperfusion glomerular filtration rate (GFR) in the rat.[10] However, they did not evaluate whether this effect was just a transitory one because GFR was not measured beyond the immediate reflow period. Klausner et al[11] reported that ANS-treated rats developed less azotemia 24 hours after ischemia than did non-neutropenic controls, suggesting there was a prolonged beneficial effect. In contrast, Paller[12] failed to show any protection from ANS pretreatment, although a beneficial effect was noted with nitrogen mustard-induced neutropenia. However, the effect of nitrogen mustard was attributed to a non-neutrophil-dependent mechanism (eg, profound weight loss), because no correlation was noted between neutrophil counts and postischemic GFR.

A potential problem with using neutrophil depletion as a probe in this type of experiment is that even severe neutropenia does not necessarily prevent neutrophils in marginated pools or in the bone marrow from entering the circulation in small numbers and participating in the injury process. For example, recent unpublished studies (personal communication, RJ Johnson, MD, Seattle, WA) demonstrated that, despite profound neutropenia (<200 cells/mm^3) induced by ANS injection, neutrophils can still participate in experimental glomerular injury, as indicated by their accumulation in glomerular capillary loops. Thus, the above discrepancies might be explained by the degree to which ANS depletes circulating as well as noncirculating neutrophil pools.

A different approach, which eliminates this potential problem, is to administer a monoclonal antibody directed against the neutrophil adherence protein (CD11b/CD18), thereby preventing them from binding to the endothelium. Adherence is generally believed to be a critical step in neutrophil-induced tissue injury because it creates a "protected microenvironment." For example, were neutrophils to release H_2O_2 without endothelial attachment, it would readily be consumed by red cell catalase, thereby preventing oxidant tissue damage. By using an antineutrophil-adherence monoclonal antibody (MoAb 60.3) in rabbits, Vedder et al[13] were able to block neutrophil sequestration in gastrointestinal and hepatic tissues after hemorrhagic shock, thereby preventing reperfusion injury and mortality.[13] Thus, these studies confirm the validity of using antineutrophil adherence antibodies as probes to assess the role of neutrophils in I-R injury.

Because this highly effective monoclonal antibody was recently made available, we sought to determine whether neutrophils play a role in renal I-R injury in the rabbit. To complement these studies we also tested whether more classic neutrophil depletion experiments using ANS protect against renal I-R injury in the rat, an issue that is open to debate.[10–12] The results of these experiments form the basis of this report.

Methods

Blockade of Neutrophil-Endothelial Adherence: Rabbit Model

The following experiments were performed using the same monoclonal antibody (MoAb 60.3) preparation and the same dosage schedule that were previously shown to protect rabbit liver, ear, stomach, and intestines against I-R injury (unpublished observations, RW),[13,14] thereby dramatically increasing survival after hemorrhagic shock.[13] MoAb 60.3 was generously supplied by John Harlan, MD, University of Washington, Seattle, WA. It is a murine IgG_{2a} antibody that recognizes a functional epitope on CD 18. It was endotoxin free, as assessed by limulus assay, and was prepared as previously described.[15]

Severe ARF Protocol

Female New Zealand white rabbits weighing 1.0 to 1.5 kg (R and R Rabbitry, Stanwood, WA) were anesthetized with intravenous ketamine (80 to 140 mg) and placed on a warming blanket to maintain body temperature between 38 and 38.5 C (assessed continuously using a rectal thermometer). Animals in the experimental group (N = 6) were pretreated with 2 mg/kg MoAb 60.3 administered intravenously 25 minutes before ischemia. Control animals (N = 6) received an equal volume (1.25 ml) of intravenous saline. Under sterile conditions the rabbits were subjected to a midline laparotomy and both renal pedicles were isolated. Fifty minutes of bilateral renal ischemia were induced with smooth vascular clamps. In the case of the left kidney the renal artery was dissected away from the vein and clamped. Right renal ischemia was induced by pedicle clamping (artery and vein) to avoid more extensive surgical dissection. After 50 minutes of vascular occlusion, the clamps were removed. Fifteen minutes into the reperfusion period the right kidney was resected. It was cut into coronal sections and preserved in 10% formalin for histologic evaluation of early reperfusion injury, as described below. At the time of nephrectomy, the right ureter was ligated to prevent intraabdominal urinary reflux from the bladder. Then, the incision was closed and the animals were allowed to recover from anesthesia. Free access to food and water was provided for the remainder of the experiment. The severity of renal injury was assessed at 24 and 48 hours postoperatively by measuring blood urea nitrogen (BUN) and plasma creatinine (CR) concentrations. At 48 hours, the animals were killed. The

left kidney was immediately resected and saved for histologic evaluation, as discussed below.

Mild ARF Protocol

To assess whether MoAb 60.3 could protect against renal injury less severe than that induced by 50 minutes of vascular occlusion, the above protocol was repeated exactly as presented except that a 38-minute ischemia period was used. Six rabbits received MoAb 60.3 pretreatment; seven saline-treated rabbits served as controls.

Neutrophil Depletion Experiments: Rats

MoAb 60.3 has no activity against rat neutrophils. Thus, to assess the role of neutrophils in I-R injury in a different animal species, the effect of neutrophil depletion on renal artery occlusion (RAO)-induced ARF in the rat was assessed. These studies were also prompted by discrepancies in the results in the literature using this approach.[10-12]

Severe ARF Protocol

Rabbit ANS[16] was generously supplied by Dr. R. J. Johnson, University of Washington, Seattle, WA. The antiserum was stored at −70 C until used. Ether-anesthetized female Sprague Dawley rats (N = 5) weighing 180 to 250 g were injected intraperitoneally with 1.25 ml rabbit ANS. A blood specimen was drawn from the tail vein and analyzed for hematocrit (HCT). Twelve to 16 hours later, the HCT was repeated, the BUN and CR concentrations were determined, and neutropenia was confirmed (<200 cells/mm^3) by a standard blood dilution method followed by a differential white blood cell count. After confirming neutropenia, the rats were anesthetized with pentobarbital (30 to 40 mg/kg), placed on a heated surgical table to maintain a constant body temperature of 37 C, and subjected to 37 minutes of bilateral RAO. After vascular reflow, the animals were sutured and allowed to recover from anesthesia. Body temperature was maintained at 37 C for 1 hour before the rats were returned to their cages. Free food and water access were provided. Twenty-four hours later, the rats were reanesthetized and killed by aortic puncture. The severity of renal injury was assessed by BUN and CR concentrations and by histology. Controls for these experiments consisted of five rats treated in an identical fashion except that nonimmune rabbit serum was substituted for anti-neutrophil serum injection.

Mild ARF Protocol

The above protocol was repeated exactly as described above except that the period of RAO was shortened to 29 minutes. Nine neutrophil depleted rats and eight nonimmune serum-injected rats were studied.

Histologic Assessments: Calculations and Statistics

All values are presented as means ± 1 SEM. BUN and CR data for individual sets of experiments for control and experimental groups were contrasted by unpaired Student's *t*-test. Significance was assessed by a *P* value of less than 0.05.

To assess renal histologic injury, coronal kidney sections including the cortex, medulla, and papilla were cut and fixed in 10% buffered formalin. Four-μm sections of paraffin-embedded tissues were stained with hematoxylin and eosin (H & E). The control and experimental kidneys for each set of experiments were contrasted. The sections were coded and the extent of ischemic injury was graded on a semiquantitative scale of 1+ to 3+ (rabbits) or 1+ to 4+ (rats) that reflected increasing degrees of ischemic damage as described in Results. The histologic scores for the control and experimental groups were contrasted by Wilcoxon Rank Sum test.

Results

Rabbit Experiments with MoAb 60.3

Functional Data

Severe azotemia was observed after 50 minutes of renal ischemia in both the control and the MoAb treated groups and it was progressive over the 24- to 48-hour period of observation (Table 1). No significant differences in the degree of BUN- or CR-concentration elevations were observed between the MoAb 60.3 and the control groups.

The 38-minute ischemic challenge produced renal injury of less severity than 50 minutes of RAO, the degree of azotemia at 24-hours approximating half of that observed in the 50-minute RAO groups. In further contrast to the 50-minute RAO experiments, 38 minutes of RAO did not produce progressive azotemia 24 and 48 hours after ischemia. Despite this much more modest ischemic damage, MoAb 60.3 conferred no functional protection (Table 1).

Table 1. *Rabbit Experiments with MoAb 60.3**

Experiment	N	24 hour BUN	24 hour Cr	48 hour BUN	48 hour Cr	Histologic injury 15 minutes	Histologic injury 48 hours
50-minute ischemia							
Control	6	84 ± 6	4.8 ± 0.2	153 ± 9	6.3 ± 0.5	2.2 ± 0.3	2.5 ± 0.3
MoAb	6	79 ± 7	4.4 ± 0.3	142 ± 17	6.2 ± 0.7	2.3 ± 0.3	2.7 ± 0.2
P		NS	NS	NS	NS	NS	NS
38-minute ischemia							
Control	6	51 ± 8	2.4 ± 0.4	63 ± 16	2.1 ± 0.4	2.2 ± 0.3	1.2 ± 0.2
MoAb	7	65 ± 10	2.2 ± 0.4	65 ± 10	2.2 ± 0.4	2.0 ± 0.0	1.3 ± 0.2
P		NS	NS	NS	NS	NS	NS

* BUN and Cr, mg/dl. The values are the mean ± 1 SEM. (For comparison, BUN, Cr concentrations for three rabbits 24 hours after right nephrectomy without left renal ischemia were 24 ± 1 and 1.1 ± 0.07, respectively.) Histologic injury was graded at 15 minutes after reflow (right kidney) and 48 hours after reflow (left kidney). Injury was scored on a semiquantitative scale from 1+ to 3+, reflecting increasing injury, as discussed in the text.

Histologic Assessments (15 minutes after reflow)

Right rabbit kidneys resected 15 minutes after RAO revealed vascular congestion in the inner stripe of the outer medulla and degenerative proximal tubular changes, largely confined to the outer medullary stripe (OMS) and the cortical/medullary rays. The tubular changes consisted of brush border membrane effacement with blebbing and flattening of proximal tubular cells that produced a cuboidal appearance. Overt necrosis was inconspicuous. The proximal tubular changes were graded as mild (1+), moderate (2+), or severe (3+). No significant difference in the extent of tubular injury was recognized between the 38-minute and 50-minute RAO control groups at 15 minutes of reflow (undoubtedly because there was insufficient time after RAO for more fulminant histologic damage to develop). MoAb treatment did not alter the severity of the histologic damage in either group. Prominent neutrophil accumulation within glomeruli or vasa rectae was not seen in either the 50-minute or 38-minute RAO groups whether or not MoAb 60.3 pretreatment had been used. Approximately 1 or 2 neutrophils were identified per high power (×400) field of outer medullary vascular bundles. Clusters of neutrophils within vasa rectae were not observed. No difference in neutrophil accumulation was observed between the MoAb and control groups.

Histologic Assessments (48 hours after reflow)

Prominent tubular necrosis was observed 48 hours after both 38 minutes and 50 minutes of RAO. The necrosis was largely confined to the OMS and cortical/medullary rays in the 38-minute RAO experiments. In the 50-minute RAO experiments, tubular necrosis was most prominent in the OMS but there was also patchy involvement of the cortical tubular segments. The extent of tubular necrosis was graded on a 1+ to 3+ scale (mild, moderate, and severe). Although a clear difference in the amount of necrosis was observed between the 50-minute and 38-minute RAO kidneys, MoAb treatment caused no apparent lessening of the damage in either set of experiments. Medullary cast formation was seen in all kidneys, the extent of which generally correlated with the amount of tubular necrosis. Patchy interstitial calcification and occasional mitotic figures were seen in some kidney sections. Medullary vascular congestion was observed in all sections but prominent neutrophil accumulation within vasa rectae was not observed, as noted above. On average, 1 or 2 neutrophils were identified per high power field of outer medullary vascular bundles; no neutrophil clusters were observed and no difference in the extent of this finding was apparent among the groups.

Rat Neutrophil Depletion Experiments

Antineutrophil serum lowered the neutrophil count to 74 ± 17 cells/mm^3 (Table 2). Of the 15 rats subjected to study, five had counts less than 25/mm^3, four had counts between 25 and 100/mm^3, four had counts between 100 and 150/mm^3, and two had counts between 150 and 200/mm^3. The HCTs were unchanged by antiserum injec-

Table 2. *Rat Neutrophil Depletion Experiments*

	N	24 hours after RAO BUN	24 hours after RAO Cr	24 hours after RAO Histology
37-minute ischemia				
Controls	5	112 ± 9	2.6 ± 0.4	3.3 ± 0.2
Neutropenic	5	111 ± 3	2.4 ± 0.3	3.4 ± 0.2
P		NS	NS	NS
29-minute ischemia				
Controls	8	36 ± 8	0.7 ± 0.07	1.5 ± 0.2
Neutropenic	9	37 ± 5	0.8 ± 0.09	1.8 ± 0.3
P		NS	NS	NS

BUN and Cr, mg/dl. Baseline values for the rats were BUN, 14 ± 1 and Cr, 0.44 ± 0.01. Histologic injury was assessed on a semiquantitative scale from 1+ to 4+, reflecting increasing injury as described in text.

tion, and platelets appeared normal in number on peripheral smear evaluation. Baseline BUN and CR concentrations were within normal limits for this laboratory (BUN, 14 ± 1; CR, 0.4 ± 0.01).

Functional Responses to RAO

Thirty-seven minutes of RAO induced severe and essentially identical degrees of azotemia in the neutropenic rats and in the controls. The 29-minute ischemic challenge caused very mild azotemia. Once again, virtually identical BUN and CR elevations were seen in the control and neutropenic groups (Table 2).

Histologic Responses to RAO

Histologic changes typical of RAO-induced ischemic injury in the rat were observed consisting of medullary vascular congestion, cast formation, and tubular necrosis, as previously described.[17] The 37-minute RAO challenge induced prominent necrosis in the OMS, with occasional foci of necrosis also seen in the cortex. In contrast, the 29-minute RAO kidneys showed only sporadic areas of OMS tubular necrosis and virtually none was seen in the cortex. There was more variation in the extent of necrosis in the rat than in the rabbit experiments. Thus, its extent was graded on a scale of 1+ to 4+ (least to most severe). Much less necrosis was observed in the 29-minute than in the 37-minute RAO kidneys. However, neutrophil depletion did not lessen the degree of necrosis after either ischemic challenge (Table 2). The degree of neutrophil accumulation within medullary vasa rectae at 24 hours was comparable with that described above for the rabbit experiments, and no obvious difference in frequency was observed among the groups. It should be noted that neutrophil counts typically begin their recovery at 18 to 24 hours after ANS injection, which possibly explains the latter result.

Discussion

Our findings strongly suggest that neutrophils and neutrophil adherence are not critical in producing *in vivo* renal I-R injury after transient arterial occlusion. We believe the strongest piece of evidence to support this conclusion to be the failure of MoAb 60.3 to protect against mild or severe ischemic ARF in the rabbit. Using two different intervals of RAO, these studies did not demonstrate any functional or morphologic benefit from a maneuver known to prevent neutrophil adherence to the endothelium. The lack of protection observed in our initial experiments using 50 minutes of RAO raised the possibility that prolonged ischemia might be causing immediate irreversible injury and obscuring any protective effect conferred by the antibody. This prompted the second set of experiments using the shorter (38 minutes) ischemia time. Preliminary studies revealed that this was the shortest ischemic interval to be capable of inducing consistent functional impairment in the rabbit, as assessed 24 hours later. That MoAb could not mitigate such a modest ischemic insult (or the more profound damage that followed 50 minutes of RAO) strongly suggests that neutrophils do not play a critical role in *in vivo* renal I-R injury in the rabbit.

A potential problem in interpreting our rabbit MoAb experiments is that we have no way of directly confirming that the antibody did, in fact, prevent neutrophil adherence within the kidney. This is because prominent intrarenal postischemic neutrophil sequestration is not routinely observed. Although one report[10] did note an increase in glomerular neutrophil counts after ischemia, this has not generally been found. For example, Bird et al[18] could not demonstrate an increase in tissue neutrophils in postischemic rat kidneys using a myeloperoxidase assay. In the present study, histologic assessments performed on right rabbit kidneys resected 15 minutes after reflow showed no prominent neutrophil sequestration. Given that neutrophils do not appear to consistently accumulate in early postischemic kidneys in amounts sufficient to permit accurate quantitation, it is difficult to prove that MoAb 60.3 blocks this process. It should be recalled, however, that the MoAb used in our experiments has previously been shown to be highly effective in preventing neutrophil sequestration and reperfusion injury in multiple extrarenal rabbit tissues, including the ear, stomach, intestines, and liver.[13,14] Thus, that it failed to prevent renal I-R injury strongly suggests that neutrophils are not critical to the renal I-R process.

Several *in vivo* and *in vitro* studies of nonrenal tissues indicate a critical role for neutrophil adherence to the endothelium in creating I-R injury.[8,19,20] Adherence is believed to produce a protected microenvironment in which ROS or other inflammatory mediators, such as proteases, can be released in such close proximity to the endothelium that they cannot be neutralized by circulating factors (eg, catalase, antiproteases). Neutrophil adherence may also influence I-R injury by occluding the microcirculation, thereby contributing to the "no-reflow" phenomenon,[20] a process believed to occur in the postischemic kidney.[21] Nevertheless, we considered the possibility that neutrophils might influence renal I-R injury merely by traversing the ischemically damaged microcirculation, at which time unneutralized inflammatory mediators might be released. Because MoAb 60.3 would not be expected to affect this process, we sought protection against I-R injury by neutrophil depletion.

In preliminary experiments we attempted to deplete rabbits of neutrophils using either nitrogen mustard or anti-rabbit neutrophil serum. However, the mustard therapy induced an extremely high mortality rate making data interpretation impossible, and we were unable to achieve a sufficient degree of neutropenia (<200 cells/mm^3) with antiserum injection to yield meaningful results. Thus, we performed our neutrophil-depletion experiments in the rat. Because the results in the literature concerning the effect of ANS-induced neutrophil depletion on ischemic ARF in the rat are inconsistent,[10-12] we believed these experiments could help resolve this controversy. Profound neutropenia was induced, with counts of less than 100 cells/mm^3 frequently observed. However, the neutrophil-depletion state conferred neither functional nor morphologic protection when either mild or severe ischemic injury was induced. These findings, therefore, exactly mimicked those that emanated from our rabbit experiments and support Paller's work in the rat.[12]

We do not believe that our results necessarily conflict with those found in the elegant studies of Linas et al[9] that demonstrated a role for neutrophils in reperfusion injury in the IPK. It should be noted that major pathogenetic differences exist between *in vivo* ischemic injury and that induced in the IPK, as pointed out by Epstein and coworkers.[22] Thus, it is possible that neutrophils affect the latter but not the former process. Furthermore, it is important to note that Linas et al evaluated ischemic injury in the immediate reflow period, and not in the early maintenance phase of ischemic ARF as we did in our experiments. It is possible, therefore, that neutrophils could have a mild, transitory effect on the postischemic kidney without influencing the severity of the I-R injury process. This would also explain why Hellberg et al[10] noted mild protection in their early reflow experiments.[10]

In conclusion, this study sought a pathogenic role of neutrophils in *in vivo* renal I-R injury by seeking functional and morphologic protection against RAO-induced ARF either by blocking neutrophil–endothelial adherence in rabbits or by neutrophil depletion in rats. Although we studied two different animal species under different experimental conditions, we observed no protection against RAO-induced ARF. The uniformity of these results provides strong evidence against the hypothesis that neutrophils are critical mediators of *in vivo* ischemic ARF. These results do not exclude a transitory neutrophil effect on immediate postischemic renal function.[9,10] However, that the severity of renal injury, as assessed during the late reflow period, was not ultimately influenced by prior anti-neutrophil interventions strongly suggests that neutrophils do not play a prominent role in renal I-R injury. We believe that this finding, viewed in conjunction with the results of previous work in this laboratory,[7] places the role of oxidant damage in postischemic kidneys in doubt.

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