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Neutrophil Fate in Experimental Glomerular Capillary Injury in the Rat

Emigration Exceeds in Situ *Clearance by Apoptosis*

Jeremy Hughes,* Richard J. Johnson,† Andrew Mooney,* Christian Hugo,† Kathy Gordon,† and John Savill*

From the Division of Renal and Inflammatory Disease, Department of Medicine, University Hospital, Nottingham, United Kingdom, and the Division of Nephrology,† Department of Medicine, University of Washington, Seattle, Washington*

Neutrophils (PMNs) and their toxic contents can injure glomeruli, but to date their fate in glomerulonephritis has been unknown. We studied glomerulonephritis induced in rats by formation of concanavalin A (Con A)/anti-Con A immune complexes on glomerular endothelial cells. PMN infiltration, which was almost exclusively confined to the lumen of glomerular capillaries, was transient, peaking at 4 hours, with only 9.0 ± 4.1% (mean ± SEM) of the maximum remaining at 24 hours. There was clear evidence of PMN apoptosis leading to phagocytosis in situ by intraluminal macrophages. However, the kinetics of leukocyte infiltration and PMN apoptosis, the preferential location at 24 hours of apoptotic PMNs within occluded capillary loops, and tracking of radiolabeled PMNs all indicated that in situ phagocytic clearance after apoptosis was the fate of a minority of PMNs, amounting to no more than one-fifth of the peak infiltrating load. Instead, the majority of infiltrating PMNs (72.9 ± 3.1%) had emigrated from inflamed glomeruli by 24 hours, apparently returning to the circulation. We conclude that PMN emigration from inflamed glomeruli is a hitherto unrecognized mechanism for regulation of PMN-mediated glomerular injury. (Am J Pathol 1997, 150:223–234)

Glomerulonephritis can resolve, but frequently persists, leading to glomerular scarring and loss of organ function. To date, little is known of the factors that determine the outcome of glomerular inflammation. Neutrophil polymorphonuclear granulocytes can damage isolated glomerular components *in vitro*, perturb isolated perfused kidneys, and injure glomeruli *in vivo* as shown by specific neutrophil depletion and reconstitution experiments in animal models of glomerulonephritis.[1–4] Neutrophils are prominent in the inflammatory infiltrate present in many types of human glomerulonephritis, particularly in lesions believed to reflect deposition of immune complexes on the endothelial side of the glomerular basement membrane, such as post-streptococcal, cryoglobulinemic, and membranoproliferative glomerulonephritides. In these conditions, neutrophils typically remain within the glomerular capillary lumen where they may injure the capillary wall by releasing reactive oxygen intermediates, toxic cationic proteins, and powerful degradative enzymes.[5–7] However, in animal models of immune complex nephritis and probably also in human glomerular disease, neutrophil infiltration is transient.[8] This indicates that there must be mechanisms available for removal of neutrophils from capillaries in the inflamed glomerulus. Nevertheless, despite potential importance in governing outcome, remarkably little is understood of the fate of the neutrophil in glomerulonephritis.

A number of fates could befall normally short-lived neutrophils recruited to the inflamed glomerulus. Neutrophils could meet their fate *in situ* and die within

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Address reprint requests to Dr. Jeremy Hughes, Division of Renal and Inflammatory Disease, Department of Medicine, University Hospital, Nottingham NG7 2UH, United Kingdom.

the glomerulus. Our earlier work indicates that neutrophils preferentially die by constitutively undergoing apoptosis (programmed cell death) *in vitro* and at inflamed sites *in vivo*.[9,10] Indeed, when neutrophils reach body cavities such as the peritoneum or alveolar spaces, there is strong histological evidence that constitutive apoptosis leading to phagocytosis by macrophages is the predominant fate of the senescent inflammatory neutrophil.[11,12] In studies of single cells obtained by enzymatic dissociation of glomeruli from rats with nephritis induced by nephrotoxic globulin, we observed that neutrophil apoptosis does indeed occur in inflamed glomeruli, leading to phagocytosis by both glomerular inflammatory macrophages and by the semi-professional phagocyte of the glomerulus, the mesangial cell.[13] However, the relevance of these observations to the fate of neutrophils remaining confined within glomerular capillaries was not clear, nor was the possible quantitative contribution of apoptosis to clearance of neutrophils from inflamed glomeruli. Nevertheless, it should be emphasized that, given the very short clearance time of apoptotic cells determined in a variety of circumstances, apparently low frequencies of apoptotic cells can equate with large-scale cell deletion by this mechanism.[14–16]

However, although constitutive apoptosis leading to phagocytic clearance *in situ* may be a strong candidate for the major mechanism eliminating neutrophils recruited to glomerular capillaries, other routes of removal are possible. In immune complex glomerulonephritis, given the propensity of neutrophils to remain in the capillary lumen, it is possible that neutrophils may detach from the capillary wall and return to the circulating pool, meeting their fate in graveyards believed to be in the spleen, liver, and bone marrow. Alternatively, given that there is evidence that macrophages may emigrate from inflamed glomeruli *via* lymphatics,[17] it is conceivable that neutrophils might also leave glomeruli by this route. Lastly, neutrophils could cross into Bowman's space and thereby pass into the urine.

The object of the current study was to assess the fate of neutrophils recruited to glomerular capillaries in an animal model of glomerulonephritis induced by *in situ* deposition of concanavalin A (Con A)/anti-Con A immune complexes on endothelial cells. Neutrophil infiltration was rapid, transient, and largely confined to the lumen of glomerular capillaries. Clear histological evidence of neutrophil apoptosis was obtained, particularly in occluded capillary loops, where phagocytosis by intraluminal macrophages was observed. By tracking re-infused neutrophils that had been irreversibly radiolabeled *ex vivo*, we found that, although many neutrophils do indeed undergo phagocytic clearance *in situ*, the majority of radiolabeled neutrophils leave the glomerulus to meet their fate elsewhere.

Materials and Methods

Reagents and Materials

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Sterile plasticware was from Falcon Plastics (Cockeysville, MD). Autoradiographic reagents were from Kodak (Rochester, NY).

Description of the Model of Glomerular Capillary Injury

Glomerular capillary injury was induced in rats by selectively perfusing the right renal artery with Con A (ICN Biomedicals, Costa Mesa, CA) followed by polyclonal anti-Con A antibody as previously described.[5] The lectin Con A binds to sugar residues on glomerular capillary endothelial cells and glomerular basement membrane glycoproteins. The subsequent perfusion of anti-Con A antibody results in antibody binding to this planted antigen with consequent *in situ* subendothelial immune complex deposition. Resultant activation of complement produces marked inflammation within the glomerular capillaries with endothelial cell damage, variable fibrin deposition, and the rapid recruitment of platelets, neutrophils, and macrophages.

Renal Artery Perfusion

The technique for inducing glomerular capillary injury with Con A and anti-Con A antibody has been described in detail previously.[5] Briefly, male Sprague-Dawley rats (Tyler Laboratories, Bellevue, WA) weighing between 200 and 300 g were anesthetized with a mixture of ketamine (50% v/v), acepromazine (15% v/v), xylazine (25% v/v), and Ringers solution (10% v/v; 0.1 ml per 100 g body weight), and a left nephrectomy was performed. The aorta was then exposed above and below the right renal and superior mesenteric arteries, which arise from opposite sides of the aorta at the same level, and the blood flow was interrupted by using clamps. The superior mesenteric artery was cannulated in a retrograde fashion with a 30-gauge needle and the right kidney was perfused using a constant infusion pump (Sage Instruments Division, Orion Research,

Cambridge, MA) at a rate of 0.75 ml/minute. The kidney was initially perfused with 0.5 ml of phosphate-buffered saline (PBS), pH 7.2, followed by 1.0 ml of PBS containing 125 μg of Con A. The cannula was flushed with 0.2 ml of PBS and the kidney was then perfused with 19 mg of polyclonal rabbit anti-Con A IgG in 0.5 ml of PBS followed by 0.2 to 0.3 ml of PBS. The preparation of the anti-Con A antibody has been described previously.[5] After the perfusions were completed, blood flow was restored to the kidney, the needle was removed, and hemostasis was obtained by the gentle application of gel foam (Upjohn Co., Kalamazoo, MI) to the puncture site. The total ischemia time for the kidney was always less than 8 minutes. The abdominal wound was then closed and the animal was placed under a heat lamp for 1 or 2 hours until recovered. Buprinorphine was administered for post-operative pain relief. This animal model was approved by the University of Washington Animal Care Committee.

Experimental Protocol

In the first study, designed to assess the time course of leukocyte infiltration and neutrophil apoptosis, animals were sacrificed 4, 8, 16, and 24 hours after the induction of Con A glomerulonephritis with four to six animals in each group. The normal contralateral left kidney was used as control. In a second study, designed to track the fate of recruited neutrophils by prior infusion of ^{111}indium-labeled neutrophils from rats of the same strain, two groups of animals were studied (four rats per group) that were sacrificed 4 and 24 hours after the induction of Con A glomerulonephritis. At sacrifice, the kidney was removed together with the draining lymph nodes. In each of the two studies, the nonmanipulated left kidney (see below) was taken as control for the inflamed kidney in each animal.

Renal Histology

Renal biopsies were fixed in methyl Carnoy's solution and embedded in paraffin. Light microscopy was performed on 4-μm sections of tissue stained with periodic acid Schiff (PAS) reagent and counterstained with hematoxylin. Immunoperoxidase staining was performed as described previously[18] using the following primary antibodies: ED1 (Bioproducts for Science, Indianapolis, IN), a murine monoclonal IgG1 to a cytoplasmic antigen present in rat monocytes and macrophages,[19] and RP-3 (gift of F. Sendo), a murine monoclonal IgM to an as yet uncharacterized cytoplasmic antigen present in rat neutrophils.[20]

Fluorescence microscopy was performed on 4-μm sections stained with propidium iodide (PI). This was performed by incubating deparaffinized sections with 100 μg/ml RNAse in PBS at 37°C for 30 minutes and then with 0.25 μg/ml PI in PBS for 5 to 10 minutes.[15] Sections were then washed in PBS and mounted in neutral glycerine jelly.

Quantitation of the number of macrophages and neutrophils per glomerular cross section and apoptotic neutrophils per 50 glomerular cross sections was performed by counting 50 representative glomeruli from each biopsy. In addition, PAS- and PI-stained tissue sections of renal biopsy material derived from a previous study[8] was examined. In this study, rats had been depleted of neutrophils by the combination of irradiation followed by the systemic administration of a rabbit polyclonal antibody to rat neutrophils before the induction of Con A glomerulonephritis. Glomerular cross sections that contained only a minor portion of the glomerular tuft were not counted.

The location of glomerular neutrophils was determined using 1-μm plastic sections stained with toluidine blue and examined by light microscopy (see below) with a minimum of 75 glomeruli being examined. Neutrophils were easily identified, and those present within the mesangium, capillary loops, or Bowman's space were counted.

Tissue for electron microscopy was fixed in half-strength Karnovsky's solution (1% paraformaldehyde and 1.25% gluteraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.0). After fixation, tissue was osmicated and dehydrated in a series of graded alcohols before being embedded in Taab resin and polymerized at 60°C overnight. Sections of 1 μm were cut and stained with toluidine blue for observation at light microscopy level. Ultrathin sections of ~100 nm were then cut, collected on nickel grids, and stained with uranyl acetate and lead citrate for observation on a Phillips CM10 electron microscope.

Preparation of ^{111}Indium-Labeled Rat Neutrophils

Neutrophils from rats of the same strain as those with nephritis were isolated and labelled *ex vivo* with ^{111}indium tropolonate in platelet-poor plasma in order to maximize cell labeling and minimize cell activation.[21] ^{111}Indium remains firmly cell bound while radiolabeling does not affect neutrophil function *in vitro* or *in vivo*.[22,23] Donor Sprague Dawley

rats underwent an exchange transfusion with Hetastarch[24] and the blood/Hetastarch mixture allowed to sediment for 40 minutes. The leukocyte-rich plasma was aspirated and neutrophils isolated as previously described.[23] Briefly, leukocyte-rich plasma was mixed with Percoll with a specific gravity that had been adjusted to 1.112 at 23°C and centrifuged at 2500 × *g* for 30 minutes at room temperature. Neutrophils were cleanly aspirated off the red cell pellet and washed twice with PBS with 10% rat platelet-poor plasma (PPP) and resuspended in PPP. Neutrophil purity, determined by light microscopic examination of May-Giemsa-stained cytospins was >96%. Neutrophils were labeled with ^{111}indium tropolonate in PPP. Thirty μl of 4 mmol/L tropolone (Fluka, Buch, Germany) stock solution in 0.9% endotoxin-free saline was mixed with 50 to 60 μCi of ^{111}indium chloride (Amersham International, Little Chalfont, UK) in a volume of 25 to 50 μl at room temperature for 1 minute. The mixture was then added to 15×10^6 to 20×10^6 neutrophils and left for 5 minutes at room temperature. After labeling the neutrophils were washed twice in PPP and resuspended in PPP for intravenous infusion. Radiolabeling did not affect neutrophil viability (trypan blue was >98% negative after labeling), and radiolabeled neutrophils underwent constitutive apoptosis *in vitro* with kinetics indistinguishable from unlabeled cells[9] (data not shown). The ^{111}indium was >95% cell associated immediately after labeling and >86% cell associated 4 to 24 hours after infusion into recipient rats. This technique gave 2.3×10^6 to 4.3×10^6 cell-associated cpm per 10×10^6 neutrophils after washing.

A total of 5×10^6 to 6×10^6 ^{111}indium-labeled neutrophils were injected into recipient rats, and glomerular capillary injury was induced 1 hour later by sequentially perfusing the right kidney with Con A followed by polyclonal anti-Con A antibody as previously outlined. This delay was based on previous experience of the recruitment kinetics of ^{111}indium-labeled rat neutrophils, which are transiently delayed in the lung before appearing in arterial blood.[24] In these experiments, the left kidney was left *in situ* so that comparisons could be made between lymph nodes draining the manipulated right and nonmanipulated left kidney. To ensure adequate recruitment of ^{111}indium-labeled neutrophils to the glomeruli, the right kidney was perfused with 150 μg of Con A followed by 25 mg of polyclonal rabbit anti-Con A antibody.

Autoradiography and Isolation of Inflamed Glomeruli

Rats were sacrificed at 4 and 24 hours after the induction of glomerular capillary injury with 4 rats in each group. Three aliquots of 1 ml of blood were taken from each animal, heparinized, and centrifuged at 300 × *g* for 6 minutes, and the plasma was removed and counted in the gamma counter. The cell pellet was washed free of plasma with PBS before being counted separately and the degree of cell association of the radioactivity calculated. At sacrifice, the inflamed kidney was removed, a renal biopsy was taken and fixed with methyl Carnoy's solution, and tissue sections were subsequently immunostained for neutrophils and macrophages as outlined above. The draining lymph nodes were removed and similarly immunostained. These sections were then coated with Kodak NTB-2 autoradiographic emulsion and exposed for 24 to 72 hours, after which they were developed in fresh D-19 and fixed with Kodak rapid fix. Some sections of renal tissue were immunostained for neutrophils and used to quantify the number of neutrophils within the glomeruli at each of the time points studied. Glomeruli were isolated from the residual renal cortex using differential sieving techniques.[25] Glomeruli were counted visually using a Fuchs-Rosenthal counting chamber (Hausser-Scientific Co., Blue Bell, PA) and glomerular ^{111}indium determined using a Prias autogamma counter (Packard Instrument Co., Downers Grove, IL). Counts were corrected to take into account the decay of the isotope between the labeling of the neutrophils and the counting of the isolated glomeruli.

Statistical Methods

All values are expressed as mean ± SEM. Statistical significance (defined as $P < 0.05$) was evaluated using the Student's *t*-test or the Wilcoxon rank sum test.

Results

In Con A Glomerulonephritis, Neutrophil Infiltration Is Transient and Largely Confined to Glomerular Capillaries

Immunostaining with the rat neutrophil-specific monoclonal antibody RP3 confirmed that the influx of neutrophils into glomeruli in inflamed kidneys was rapid but short lived. Neutrophil infiltration peaked at

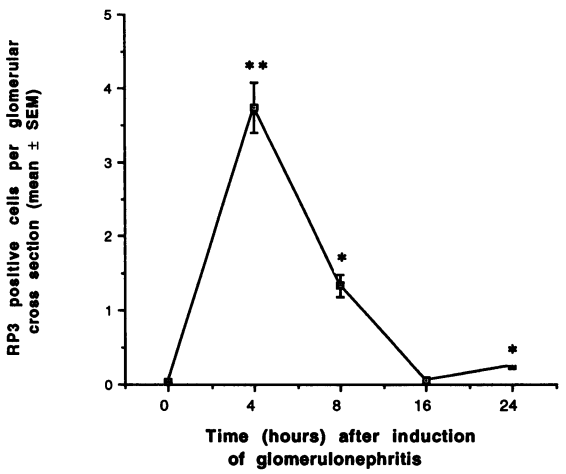


Figure 1. *Neutrophil infiltration is transient; time course study of RP3-positive neutrophil infiltration in Con A glomerulonephritis in rats. See text for further description. Mean ± SEM; n = 4 at 4 hours, but n = 6 at all other time points.* *P < 0.05; **P < 0.005 versus *control animals.*

3.74 ± 0.34 (mean ± SEM) at 4 hours but rapidly fell thereafter (Figure 1); in normal control contralateral kidneys, neutrophil infiltration assessed by RP3 staining tissue never exceeded 0.04 per glomerular cross section (data not shown). The vast majority of neutrophils remained within the glomerular capillaries as shown in Table 1. At no time point were neutrophils observed in Bowman's space.

Neutrophil Apoptosis and Phagocytosis by Intraluminal Macrophages Occurs in Inflamed Glomeruli

Neutrophils are programmed to die by apoptosis.[9] Unequivocal evidence for apoptosis of neutrophils in Con A glomerulonephritis was found at the light and electron microscopic level. First, typical nuclear features of apoptosis of neutrophils were detected at the light microscopic level in occasional neutrophils immunostained with RP3 (Figure 2). Oil immersion microscopy of both PAS and propidium iodide (PI)-stained sections also revealed apoptotic cells (Figure 2) with two or more masses of condensed chromatin that were apparently identical in appearance to apoptotic neutrophils detected in previous *in vitro* and *in vivo* studies.[9–13] Furthermore, such cells were always detected in positions consistent with glomerular capillaries to which neutrophil infiltration was effectively limited (Table 1). The reliability of these techniques for detecting neutrophil apoptosis was checked by studying renal tissue from neutrophil-depleted animals with Con A glomerulonephritis in which there was still immune complex deposition with significant accumulation of mononuclear cells and platelets together with proteinuria despite a marked reduction in neutrophil infiltration[8]; no apoptotic cells were found in many sections (data not shown). Interestingly, despite our previous success in correlating PAS and PI counts of mesangial cell apoptosis with terminal deoxynucleotidyl transferase-nick end labeling,[16] we were unable to achieve reliable and selective labeling of apoptotic neutrophils despite repeated attempts, probably because of rapid autolysis of neutrophil DNA in these studies. Second, from as early as 4 hours, electron microscopy of renal tissue frequently demonstrated intraluminal macrophages with phagolysosomes containing condensed chromatin typical of apoptotic cells undergoing degradation, a process known to be extremely rapid.[9–11] Furthermore, occasional grids at all time points revealed ingested apoptotic cells at early stages of degradation such that neutrophil granules were apparent (Figure 3).

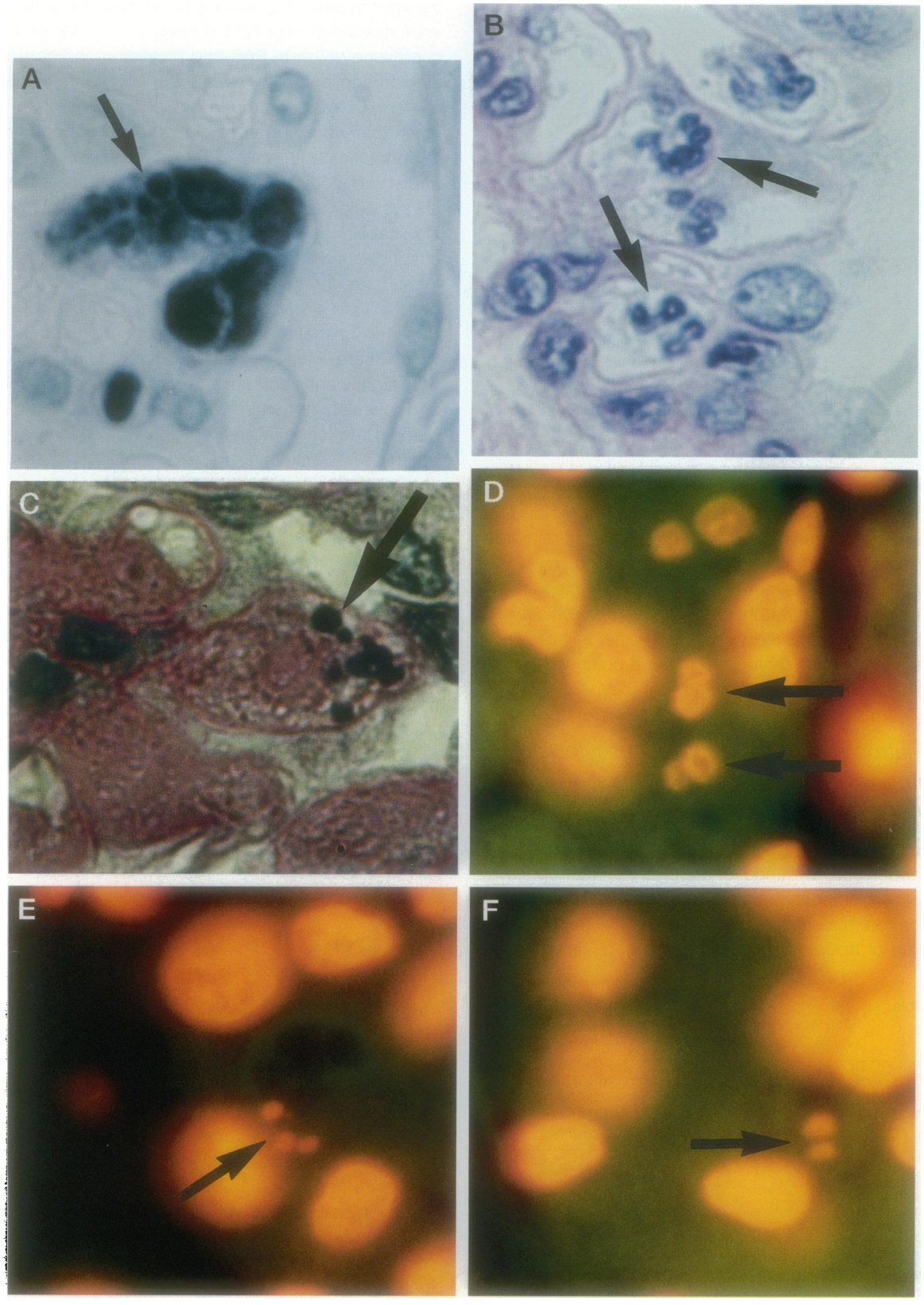
Table 1. *Intraglomerular Location of Neutrophils at Various Time Points during Con A Glomerulonephritis.*

Time	% intracapillary	% extracapillary	Total number of neutrophils (and glomeruli) counted
4 hours	99.9	0.1	930 (292)
8 hours	100	0	152 (96)
16 hours	98.8	1.2	86 (75)
24 hours	98.3	1.7	119 (210)

In all glomeruli studied, a total of four extracapillary neutrophils were identified that were in a mesangial location whereas no neutrophils were identified within Bowman's space. As we focused upon the 4- and 24-hour time points in later experiments using radiolabeled neutrophils to track neutrophil fate, a greater number of glomeruli were counted at these particular time points.

Time Course and Site of Neutrophil Apoptosis

Counts of the time course of neutrophil apoptosis by both PAS and PI yielded comparable data; for convenience, only the former are shown (Figure 4). In comparison with the time course of neutrophil infiltration (Figure 1), it can be seen that the number of apoptotic neutrophils present peaked later, at 8 hours, and then slowly fell. However, it is important to note that the proportion of neutrophils with features of apoptosis progressively increased from 1.6% at 4 hours to reach a peak of 17.6% at 24 hours. Furthermore, by 24 hours, apoptotic neutrophils were typically seen within occluded glomerular capillary loops (Figure 2). These data implied that deletion by apoptosis operated mainly in disposal of neutrophils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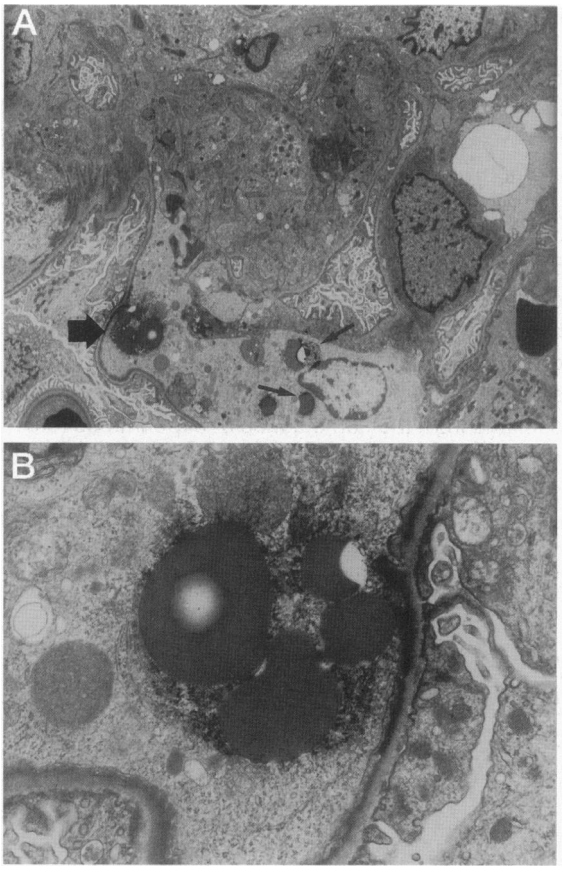

Figure 3. *Electron microscopy of Con A glomerulonephritis at 48 hours.* A: *Intact neutrophil* (broad arrow) *that has been phagocytosed by a macrophage within a capillary loop. Numerous phagolysosomes containing dense chromatin are apparent* (thin arrows) *within the macrophage, indicating the previous ingestion of the apoptotic cell(s). (Magnification, ×10,000).* B: *The intact ingested cell at higher magnification. The nuclear chromatin is condensed into several spheres characteristic of apoptosis whereas the cytoplasm contains numerous granules, thereby confirming the cell is of neutrophil origin. Magnification, ×36,000.*

remaining in glomeruli, only making a major contribution to neutrophil clearance in the later stages of the 24-hour time course studied. Furthermore, our findings also implied that other mechanisms were involved in the rapid reduction in neutrophil infiltration that began as early as 4 hours after immune complex formation. It was therefore important to determine whether recruited neutrophils were able to leave inflamed glomeruli.

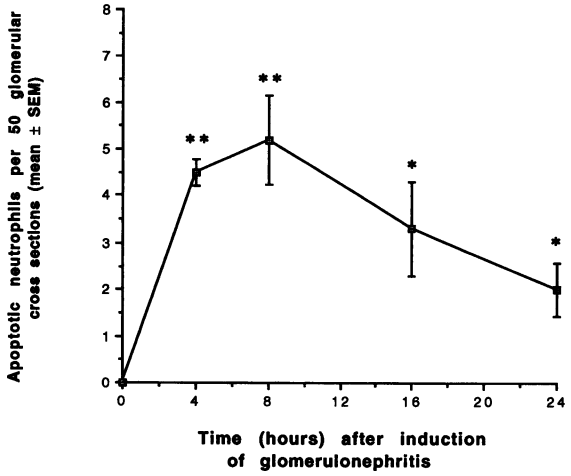

Figure 4. *Time course study of neutrophil apoptosis in Con A glomerulonephritis in rats. Apoptotic neutrophil number per 50 glomerular cross sections in PAS-stained material is shown. See text for further description. Mean ± SEM; n = 4 at 4 hours, but n = 6 at all other time points.* *P < *0.05;* **P < *0.005* versus *control animals.*

Infusion of Radiolabeled Cells Indicates that Most Neutrophils Have Left Glomeruli by 24 Hours

Neutrophil fate was tracked by intravenous infusion of ^{111}indium-labeled neutrophils. Radiolabeling with ^{111}indium tropolonate has been demonstrated not to affect the kinetics of circulation of neutrophils or their *in vivo* responsiveness.[22] In addition, the ^{111}indium remains tightly associated with the infused neutrophils for up to 48 hours.[26] In keeping with previous studies in which this technique has been used to assess neutrophil kinetics in inflammation of other organs, inflamed glomeruli clearly exhibited labeled cells marked by discrete foci of autoradiographic grains at all time points examined[22,26] (Figure 5). At 4 hours, the peak of neutrophil infiltration, comparison of the glomerular radioactive counts obtained from the nephritic kidney and the normal kidney showed that just over 90% of radioactive counts were localized to the acutely inflamed glomeruli of the right kidney; by 24 hours, counts in the nonperfused normal left kidney had fallen to low levels consistent only with the circulating blood pool. Therefore, at 4 hours, noninflammatory margination represented, at most, 10% of counts in the nephritic

Figure 2. *Neutrophil apoptosis in Con A glomerulonephritis.* A: *Light microscopy (×1000) of an RP3-immunostained section from a 4-hour renal biopsy. A number of RP3-positive neutrophils are evident within a glomerular capillary lumen, one of which* (arrow) *is clearly apoptotic.* B *and* C: *Light microscopy (×1000) of PAS-stained sections.* B *(4-hour biopsy) shows normal neutrophils* (arrow) *within a glomerular capillary loop. In* C *(24-hour biopsy), a small, intraluminal bilobed apoptotic cell* (arrow) *is shown within a thrombosed, occluded glomerular capillary loop, which is the typical location of apoptotic neutrophils at later time points. Note the dense chromatin condensation.* D *to* F: *Oil-immersion fluorescence microscopy (×1000) of PI-stained sections. In* D *(4-hour biopsy), normal neutrophils are evident* (arrows). *Note the open pattern of nuclear staining.* E *(4-hour biopsy) and* F *(24-hour biopsy) demonstrate apoptotic neutrophils* (arrow) *that exhibit typical condensed nuclear chromatin.*

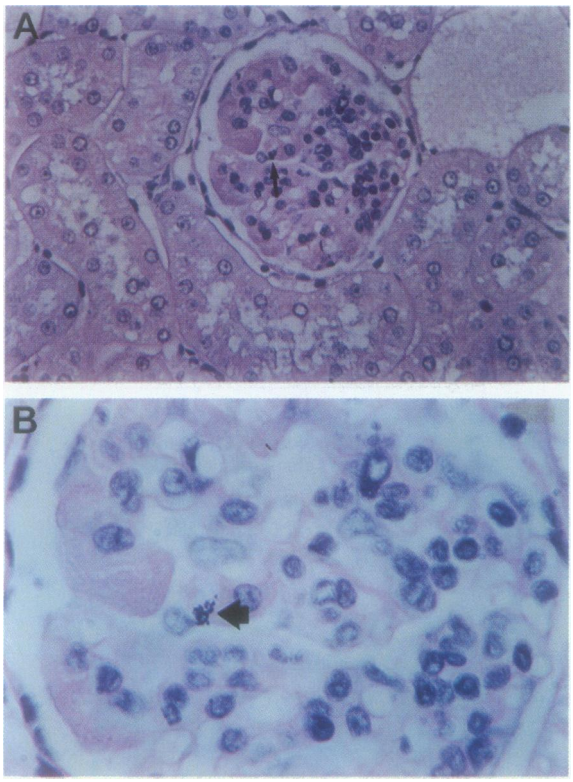

Figure 5. *Radiolabeled neutrophils are recruited to the inflamed glomerulus. Autoradiographs demonstrate that autoradiographic grains are localized to a glomerular capillary loop.* A: *Light microscopy (×400) of PAS-stained material from a renal biopsy taken at 4 hours after the induction of Con A glomerulonephritis. The* arrow *shows a typical focus of autoradiographic grains overlying a capillary loop. Note low background staining.* B: *Same glomerulus at higher magnification (×1000) showing the autoradiographic grains discretely overlying a neutrophil* (arrow).

kidney, the vast majority of counts representing ^{111}indium-labeled neutrophils specifically recruited to inflamed glomerular capillaries. At 24 hours after induction of glomerulonephritis, when noninflammatory margination was minimal, radioactive counts of glomeruli isolated from the inflamed kidney showed that 27.1 ± 3.1% (mean ± SEM) of neutrophil-associated radioactive counts present at 4 hours were still within glomeruli (Figure 6). Although interpretation of the comparison requires caution, it was notable that this figure was approximately threefold greater than neutrophil infiltration at 24 hours assessed by counting of specifically immunostained sections from the same series of experiments (9.0 ± 4.1% of neutrophil number at the 4-hour peak of infiltration). This implied that up to two-thirds of radioactive counts present at 24 hours corresponded to neutrophils that were no longer histologically recognizable.

Macrophages Bearing Radiolabeled Cell Debris Are Present in Glomeruli at 24 Hours

Macrophages are known to be capable of rapid uptake of apoptotic neutrophils at inflamed sites, quickly degrading the ingested cells beyond histological recognition.[9] It therefore appeared likely that invisible radiolabeled neutrophils remaining in glomeruli at 24 hours had been taken up and degraded by macrophages. We confirmed previous data on macrophage influx in the Con A model, finding a progressive increase in glomerular ED1$^+$ cells from

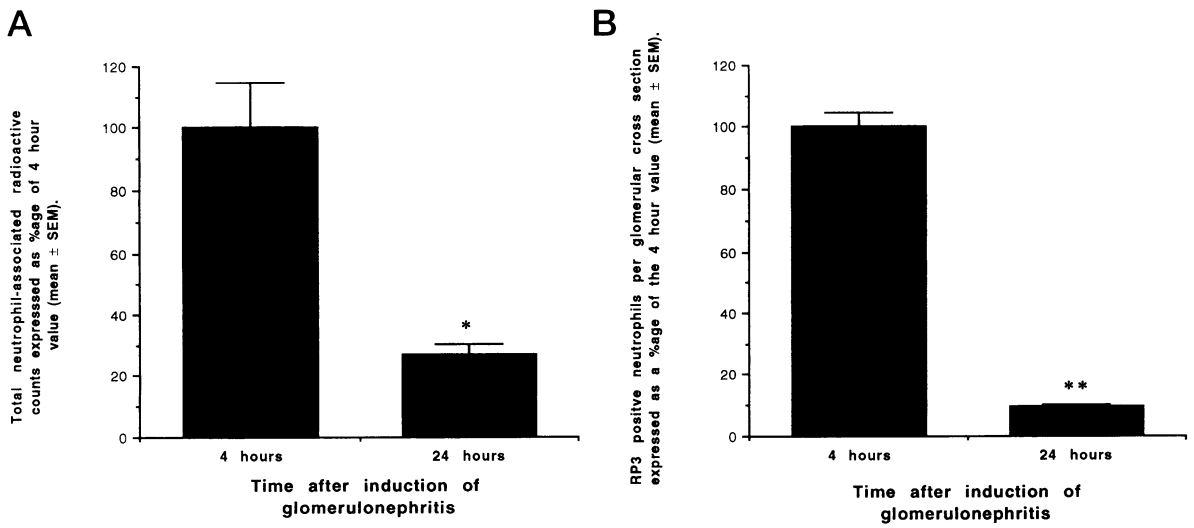

Figure 6. *Approximately one-fifth of neutrophils meet their fate within the glomerulus. Neutrophil-associated radioactive counts* (A) *are shown at 4 and 24 hours, and histologically detectable RP3-positive cells per glomerular cross section* (B) *are shown at 4 and 24 hours after induction of Con A glomerulonephritis. Data are expressed as a percentage of the 4-hour value, which is represented as 100%. At 4 hours, the SEM represents the variability of replicate observations around the mean, standardized to 100%. See text for further description. Mean ± SEM; n = 4.* *$P < 0.05$; **$P < 0.005$ versus 4-hour values.

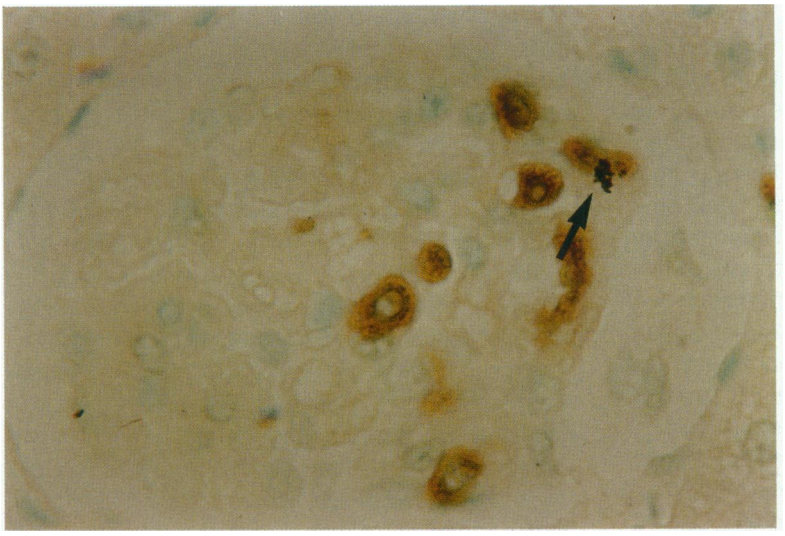

Figure 7. *Glomerular macrophages ingest radiolabeled neutrophils. Light microscopy of (×1000) an autoradiograph of renal tissue (24-hour biopsy) immunostained with the MAb ED1, which is specific for the rat macrophage. A discrete focus of autoradiographic grains localized to a glomerular ED1-positive cell is evident.*

0.71 ± 0.05 (mean ± SEM) per glomerular cross section at 0 hours through 2.3 ± 0.14 at 4 hours to 6.9 ± 0.33 at 24 hours. If phagocytosis by macrophages accounted for the demise of neutrophils meeting their fate *in situ*, then, as highlighted above, we would have expected approximately two-thirds of glomerular radioactive counts present at 24 hours to be within macrophages. Pooling our data from all animals (n = 8) we found that at 24 hours exactly 50% of discrete autoradiographic foci were associated with $ED1^+$ glomerular macrophages (Figure 7).

Radiolabeled Neutrophil Clearance via *Lymphatics is Minimal*

Radioactive counts of perihilar lymph nodes ipsilateral and contralateral to the Con A/anti-Con A injured kidney revealed very low levels of radioactivity that were not significantly different. This was strong evidence against large-scale neutrophil clearance *via* lymphatics although occasional RP3-positive neutrophils were seen in histological sections of perihilar nodes. Interestingly, despite the low total counts, autoradiography revealed occasional cells marked by discrete foci of grains. Pooling data from all animals, 100% of such foci were found to overlie $ED1^+$ macrophages.

Discussion

Neutrophils and their toxic contents have been implicated in the initiation and progression of glomerular injury. Although previous studies of experimental glomerulonephritis have documented transient infiltration of inflamed glomeruli by large numbers of neutrophils,[8] no previous report has quantified the fate of normally short-lived neutrophils recruited to the glomerulus. It would seem particularly important to determine what proportion of neutrophils meet their fate in glomeruli as glomerular tissue might be exposed to leakage of all of the histotoxic contents of this load of dying cells. Furthermore, it would be important to examine how neutrophils dying *in situ* are cleared, as constitutive apoptosis leading to phagocytic clearance by macrophages appeared to be the predominant neutrophil disposal mechanism operating in the peritoneum and alveolar spaces, efficiently deleting senescent neutrophils while protecting surrounding tissues against uncontrolled release of toxic contents from dying granulocytes.[11,12] However, glomeruli might also be protected against injury threatened by leakage of proinflammatory contents from dying granulocytes if inflammatory neutrophils were to leave the glomerulus and meet their fate elsewhere.

In the current study, we addressed these issues in a rat model of unilateral glomerular injury induced by *in situ* formation of Con A/anti-Con A immune complexes, in which we demonstrated neutrophil infiltration to be transient (<10% of the 4-hour peak being histologically detectable at 24 hours) and almost exclusively confined to the lumen of injured glomerular capillaries. Clear histological evidence of neutrophil apoptosis was observed as early as 4 hours after initiation of glomerular injury with *in situ* phagocytic clearance by intraluminal macrophages prominent at 24 hours. Indeed, at this time point, apoptotic neutrophils were typically located in occluded capillary loops, implying that phagocytic clearance after apoptosis might be particularly important for neutro-

phils trapped in glomeruli at 24 hours. Furthermore, the time course of neutrophil apoptosis expressed as a proportion of histologically detectable neutrophils suggested that neutrophil apoptosis leading to phagocytic clearance might become a quantitatively important mechanism for neutrophil removal from glomeruli only in the later stages of the 24-hour time course studied. This possibility was supported by the time course of macrophage infiltration, with threefold more ED1$^+$ monocyte/macrophages being available for phagocytic clearance at 24 hours than at 4 hours. Indeed, work on human monocytes has emphasized that these must mature into macrophages before they can take up apoptotic cells.[27] Although in the rat monocyte/macrophage series there are no known immunohistochemical markers of competence for phagocytosis of apoptotic cells, it seems likely from the work on human monocytes that many ED1$^+$ cells detected at 4 hours after injury might not have acquired phagocytic capacity for apoptotic cells, thereby further reducing the opportunities for phagocytic clearance of neutrophils early in the time course. Therefore, it appeared likely that reduction in neutrophil infiltration earlier in the time course might reflect emigration of neutrophils from the inflamed glomerulus rather than phagocytic clearance.

To determine the proportions of neutrophils either leaving or being retained within glomeruli during the 24 hours after Con A/anti-Con A immune complex formation, we tracked neutrophil fate by infusing rats with neutrophils that had been purified from rats of the same strain and irreversibly radiolabeled *ex vivo*. Radiolabeled neutrophils were infused intravenously 1 hour before induction of unilateral glomerular injury. By 24 hours, 27.1 ± 3.1% of the gamma counts present at 4 hours remained in glomeruli isolated from the injured kidney. Because the number of glomerular neutrophils seen in immunostained sections from the same series of experiments was only 9.0 ± 4.1% of the 4-hour peak, this implied that as many as two-thirds of retained neutrophils were no longer histologically detectable, equating to approximately 18% of the 4-hour peak. Neutrophil apoptosis leading to swift uptake and rapid degradation by glomerular macrophages represented a probable fate for such histologically undetectable neutrophils particularly as at 24 hours we found that 50% of discrete autoradiographic foci of neutrophil-derived radiolabel overlaid ED1$^+$ macrophages in autoradiographs of glomeruli. Furthermore, 17.6% of histologically detectable neutrophils remaining in glomeruli at 24 hours were apoptotic. Therefore, we conclude that, in a model of glomerular capillary injury in which greater than 98% of detectable neutrophils remain within glomerular capillaries, up to one-fifth of neutrophils meet their fate within glomeruli, undergoing apoptosis that leads to phagocytosis by macrophages.

However, the most striking finding in this study was that 72.9% of radiolabeled neutrophils infiltrating inflamed glomeruli at 4 hours had emigrated from glomeruli by 24 hours. Lymph nodes draining the inflamed kidney revealed very occasional macrophages containing neutrophil-derived radioactivity, suggesting that a few neutrophils leaving glomeruli between 4 and 24 hours may have been ingested in glomeruli by macrophages that subsequently emigrated from the kidney *via* the lymphatic system. Alternatively, the small number of neutrophils detected in draining lymph nodes suggests that a few neutrophils emigrating by this route might have undergone apoptosis and then phagocytosis within the lymphatics. Nevertheless, the very low levels of radioactivity within draining lymph nodes, which was not significantly different between injured and control kidneys, argues very strongly against neutrophil clearance *via* the lymphatic system being a quantitatively important route of neutrophil elimination. Furthermore, in our extensive electron and light microscopic studies, neutrophils were never seen traversing the glomerular basement membrane or within Bowman's space, indicating that large-scale removal *via* the urine was most unlikely. Instead, we suggest that the majority of neutrophils leaving the glomerulus probably do so by detaching from the endothelium and returning to the blood stream to meet their fate elsewhere, most probably in the liver and spleen.

However, ^{111}indium labeling of infused neutrophils is the only validated technique currently available to track neutrophil fate in the rat. It is not possible by means of this technique to demonstrate definitively that neutrophils leaving glomeruli do indeed meet their fate in the liver or spleen. This is because only a very small fraction (0.01 ± 0.002%, mean ± SEM) of the total number of intravenously injected radiolabeled neutrophils were recruited to the nephritic glomeruli of the inflamed right kidney at the 4-hour time point when neutrophil infiltration was maximal. The vast majority of the injected radiolabeled neutrophils circulated normally and therefore met their physiological fate in the liver and spleen. The resultant high radioactivity in the liver and spleen precludes detection of the relatively much smaller numbers of neutrophils that probably accumulate at these sites after leaving inflamed glomeruli.

Lastly, our study has the important implication that future studies should address mechanisms by which neutrophils detach from the glomerular capillary wall as this may yield important insights into how neutrophil-mediated tissue injury in glomerulonephritis is regulated. It can be speculated that apoptosis might be one such mechanism as apoptotic neutrophils exhibit marked deficiencies in adherence and migration[28] (J. Savill, unpublished observations). In this case, the small proportion of apoptotic neutrophils seen at early time points might nevertheless have major significance for neutrophil elimination as apoptosis in adherent neutrophils could lead to immediate detachment from glomerular endothelium and disappearance from the glomerulus in the bloodstream with a half-time even shorter than that for phagocytic clearance. However, it is clear that future studies will need to characterize routes of neutrophil emigration from inflamed glomeruli before the mechanisms responsible can be studied.

To conclude, this study of the Con A/anti-Con A model of immune complex glomerulonephritis is the first to quantitate neutrophil fate in this disorder. In keeping with studies of neutrophil disposal at other inflamed sites, there was clear evidence of neutrophil apoptosis leading to potentially injury-limiting phagocytosis by macrophages. However, the data indicated that apoptosis resulting in phagocytic clearance *in situ* is only a major route of neutrophil elimination in the later stages of the 24 hour time course studied, accounting for disposal of no more than one-fifth of recruited neutrophils by 24 hours. Instead, the majority of neutrophils recruited at 4 hours had left the glomerulus by 24 hours, probably *via* the blood. Our experiments indicate that new insights into control of neutrophil-mediated glomerular injury will come from studies of mechanisms by which neutrophils emigrate from inflamed glomeruli.

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