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Acute passive anti-glomerular basement membrane nephritis in P-selectin-deficient mice

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Acute passive anti-glomerular basement membrane nephritis in Pselectin-deficient mice. P-selectin present on surfaces of activated endothelium and platelets mediates neutrophil-endothelial and neutrophilplatelet interactions. The role of P-selectin in vivo was examined in a model of acute passive anti-GBM nephritis in P-selectin-deficient and wild-type mice which was induced by intravenous injection of anti-GBM serum. There were two major differences between P-selectin-deficient and wild-type mice. Firstly, mutant mice had approximately two fold more glomerular PMNs and albuminuria than wild-type animals at the peak of neutrophil influx and proteinuria. Secondly, Lipoxin A_4 (LXA₄), an cicosanoid which inhibits leukocyte-endothelial adhesion in vitro, and is generated primarily by transcellular biosynthetic routes during P-selectinmediated platelet-PMN interaction [1], was approximately 60% of wild type levels in nephritic kidneys of P-selectin-deficient mice. Injection of wild-type platelets into P-selectin-null mice restored LXA₄ to wild-type levels. The corresponding PMN influx approximated PMN levels in wild-type mice receiving platelets but urine albuminuria remained higher. Although these two P-selectin-dependent events cannot be directly linked, our results point to the importance of considering both platelet and endothelial P-selectin in determining the cellular events in inflammation.

Leukocyte adhesion to endothelium in inflammation is mediated largely by the interaction of complementary adhesion molecules on leukocytes and endothelial cells. The avidity, density and surface expression of such molecules are regulated by cytokines, chemoattractants and other endogenous mediators. Adhesion molecules of the selectin, immunoglobulin, and integrin family have been identified and their roles in various phases and types of inflammation have been studied extensively both in vitro and in vivo [2]. The recent development of genetically engineered animals deficient in adhesion molecules has provided powerful tools for the dissection of the roles of these ligands in acute and chronic inflammatory responses [3].

The selectins are present on activated endothelium and platelets (P-selectin), leukocytes (L-selectin), and cytokine-activated endothelium (E-selectin). Selectins promote leukocyte rolling on venular endothelium, which is a prerequisite to firm adhesion and diapedesis mediated by integrins and members of the immu-

noglobulin superfamily [4]. In P-selectin-deficient mice, initial studies have shown that leukocyte rolling is compromised and neutrophil extravasation in thioglycollate-induced peritoneal inflammation is delayed [5]. P-selectin is also present on activated platelets where it mediates leukocyte-platelet adhesion in vitro [6] and may be important for leukocyte recruitment into developing thrombi and subsequent fibrin deposition in vivo [7].

P-selectin is also a key mediator of several other neutrophil functional responses, including enhanced biosynthesis of lipoxin A_4 (LXA₄) [1], an arachidonate derived lipid mediator. LXA₄ is generated in modest amounts in suspensions of cytokine primed PMNs in vitro predominantly via the dual oxygenation of arachidonic acid by two lipoxygenases, 5- and 15-lipoxygenase [8]. Lipoxin generation is dramatically increased in vitro if PMNs are stimulated in the presence of platelets. In this setting, platelets that lack the enzymatic capacity to form LXA₄ from arachidonic acid, convert the PMN-derived 5-lipoxygenase product, leukotriene A₄ (LTA₄), to LXA₄ via platelet 12-lipoxygenase [8]. This transcellular LXA₄ generation is facilitated by P-selectin-mediated platelet-PMN adhesion [1]. LXA, has recently been shown to be a potential inhibitor of neutrophil, endothelial adhesion in vitro and in vivo [1, 9, 10] and may be a potent stop signal for PMN trafficking [11].

Studies of experimental glomerulonephritis (GN) in laboratory animals have yielded important insights into the pathogenesis of autoimmune glomerular diseases. In models of immune complexmediated glomerulonephritis, neutrophils (PMNs) are recruited via complement-dependent and -independent mechanisms, and are well defined effectors of glomerular injury and proteinuria through the release of oxygen radicals, proteinases, lipoxygenase products, and other inflammatory mediators. Studies with antibodies to adhesion molecules have confirmed that leukocyte and endothelial adhesion molecules are important mediators of neutrophil recruitment in various models of experimental glomerulonephritis [12].

In the present study, we used P-selectin-deficient mice to study the possible roles of P-selectin in the acute heterologous phase of anti-GBM nephritis. This model has been previously examined in detail [13-15], is associated with rapid neutrophil influx and proteinuria, and represents a reproducible, quantitative model to assess the role of P-selectin in mediating acute passive anti-GBM nephritis.

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Methods

Animals and experimental protocol

P-selectin-deficient and wild-type mice, a 129Sv and C57B1/6 mixed strain generated previously, were maintained and bred in a conventional animal facility at Center for Cancer Research, Massachusetts Institute of Technology. Male and female animals 6 to 8 weeks of age were used. One hour prior to the initiation of the experimental protocol, urine was collected from all animals to determine control levels of albumin in the urine. Nephritis was induced by tail vein injections of 0.25 ml of serum containing rabbit anti-GBM antibody. The immunization procedure and preparation of the antibody have been described previously [13]. The small dose used was chosen so as to give significant proteinuria and glomerular neutrophil influx in the early heterologous phase. This dose does not lead to glomerular thrombosis and necrosis and is associated with an insignificant autologous phase. Larger doses induce necrosis and thrombosis, and subsequent proliferation and mesangial sclerosis [14]. Animals were then placed separately in cages and monitored for urinary excretion which was collected into an eppendorf tube. Thirty minutes, one hour, two hours, eight hours or 24 hours after the anti-GBM antibody injection, animals were euthanized to obtain renal tissue. Additionally, for the 30 minute, one hour and two hour timepoints prior to euthanasia, animals were lightly etherized and blood was collected by retroorbital venous plexus sampling. For platelet isolation, blood from wild-type mice was collected in EDTA and pooled. Platelets were collected by filtering platelet-rich plasma through a Sepharose 2B (Pharmacia, Piscataway, NJ, USA) column equilibrated with 25 mm Pipes, 137 mm NaCl, 4 mm KCl and 0.1% wt/vol glucose at pH 6.8. Five to 8×10^6 platelets per gram of animal (approximately 1.1 to 1.7×10^8 per animal) was injected 15 to 20 minutes prior to injection of anti-GBM antibody. This represents approximately 5% of the animals total platelet count.

Histopathology

Normal and glomerulonephritic kidneys were prepared for light, immunofluorescence and electron microscopy by standard techniques, as previously described [1]. Glomerular PMN infiltration was assessed by the dichloroacetate esterase reaction, as previously reported [16], and expressed as PMN per glomerular cross section. On average, 150 glomeruli were counted per animal. Semiquantitative assessment of glomerular binding of the rabbit anti-GBM was monitored by indirect immunofluorescence using a FITC-tagged secondary antibody (goat anti-rabbit, Vector Laboratories, Inc., Burlingame, CA, USA).

LXA, and LTB, quantitation

Lipoxin A_4 and leukotriene B_4 levels were monitored in renal homogenates (Polytron PT-MR3000, 3 strokes, 4°C), as an index of LXA₄ and LTB₄ generation, using specific and sensitive ELISAs (Oxford Biomedical Research, Inc., Oxford, MI, USA). The LXA₄ ELISA has a lower limit of detection for LXA₄ of 290 pg/ml and cross reactivities with other lipoxygenase products as follows: LXB₄ 1%; 5(S), 6(R)-DiHETE < 5%, 15-HETE 0.1%; 5-HETE, 12-HETE, leukotrienes, each < 0.1% [17]. LTB₄ ELISA has a lower limit of detection for LTB₄ of 550 pg/ml and cross reactivities with other oxygenated metabolites of arachidonic acid as follows: 5(S), 12(S)-DiHETE 6%; 6-trans-LTB₄ 4%; 20-hy-

droxy-LTB₄ 0.5%; 5(S)-HETE 0.15%; 20-carboxy-LTB₄, arachidonic acid, 12(S)-HETE and 12(R)-HETE, each < 0.1%; and 15-HETE, leukotrienes, prostaglandins, thromboxane B₂, each < 0.01%. The sensitivity of the assay allows one to detect LXA₄ in kidneys from a single mouse, which is difficult using HPLC with UV, or other methods of detection because of the small quantities generated in murine kidneys and the inevitable loss of some LXA₄ and isomerization to all-trans-LXA₄ during the extraction procedure.

Determination of urinary albumin and creatinine concentration

To determine albuminuria, a double-sandwich ELISA was performed. Wells of microtiter plates were coated overnight with 5 μ g/ml of affinity-purified goat anti-mouse albumin (Bethyl Laboratories, Montgomery, TX, USA) diluted in 0.05 m carbonate buffer, pH 9.5. The wells were blocked with PBS containing 0.05 ml/100 ml of Tween 20 (PBS-T) and 0.5 g/100 ml BSA (Sigma Chemical Co., St. Louis, MO, USA). The plates were washed three times with PBS-T and incubated with samples or standards diluted in PBS-T + 0.5 g/100 ml BSA (PBS-T-BSA) for two hours at room temperature. After washing the plates, the wells were incubated for two hours at room temperature with horseradish peroxidase-labeled, affinity-purified goat anti-mouse albumin (Bethyl Laboratories) diluted in PBS-T-BSA. The bound peroxidaselabeled antibody was detected using o-phenylenediamine as the substrate and the absorbance read at 490 nm with a microtiter plate reader. The assay had a detectable range of 10 to 1000 ng/ml of albumin. Creatinine was quantitated spectrophotometrically using a commercially available kit (Sigma Chemical Co.). To standardize urine albumin excretion for glomerular filtration rate, proteinuria was expressed as μg of urinary albumin per mg of urinary creatinine.

Blood sampling

Blood from retroorbital venous plexus sampling was collected in 5 μ l of 0.5 M EDTA. Blood was subjected to a Coulter count to determine the total number of leukocytes. Blood smears were prepared and stained with Wright-Giemsa stain and a differential PMN count was performed.

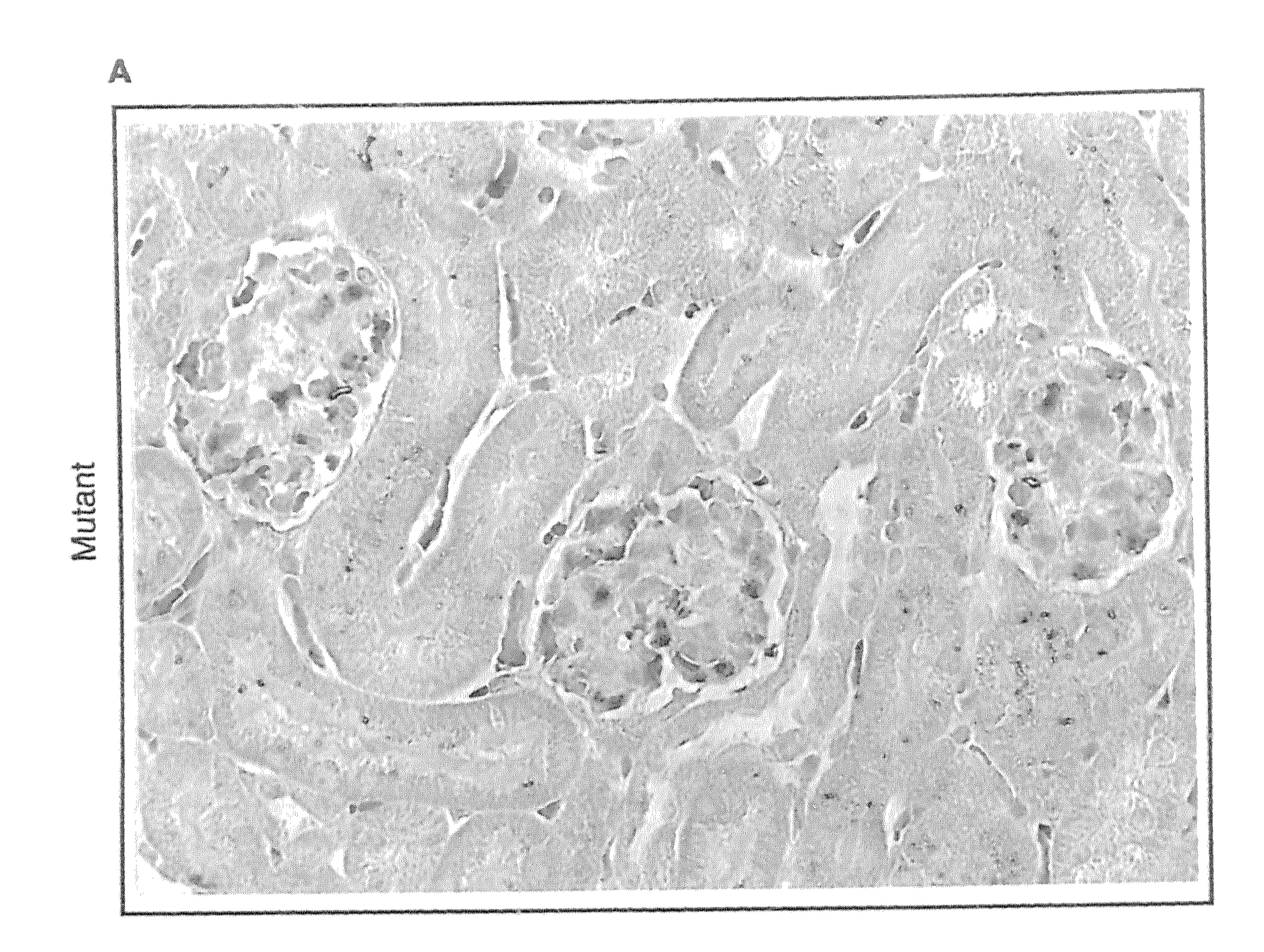
Statistical analysis

The mean and SEM were calculated on all the parameters determined in this study. Unpaired Student's t-test were performed for unpaired data and P values < 0.05 were accepted as statistically significant. Linear regressions were performed to examine the significance of the relationship between neutrophils per glomerular cross section and circulating neutrophils per ml of blood.

Results

Effect of P-selectin-deficiency on glomerular PMN infiltrate and urinary protein excretion

Acute GN was induced in P-selectin-deficient and wild-type mice using a previously characterized rabbit anti-mouse anti-GBM antibody [13]. The acute heterologous phase of this model is characterized by complement-independent glomerular PMN accumulation and proteinuria. PMN influx and proteinuria peaked at two and eight hours, respectively, after injection of antibody in both P-selectin-deficient and wild-type mice, and had



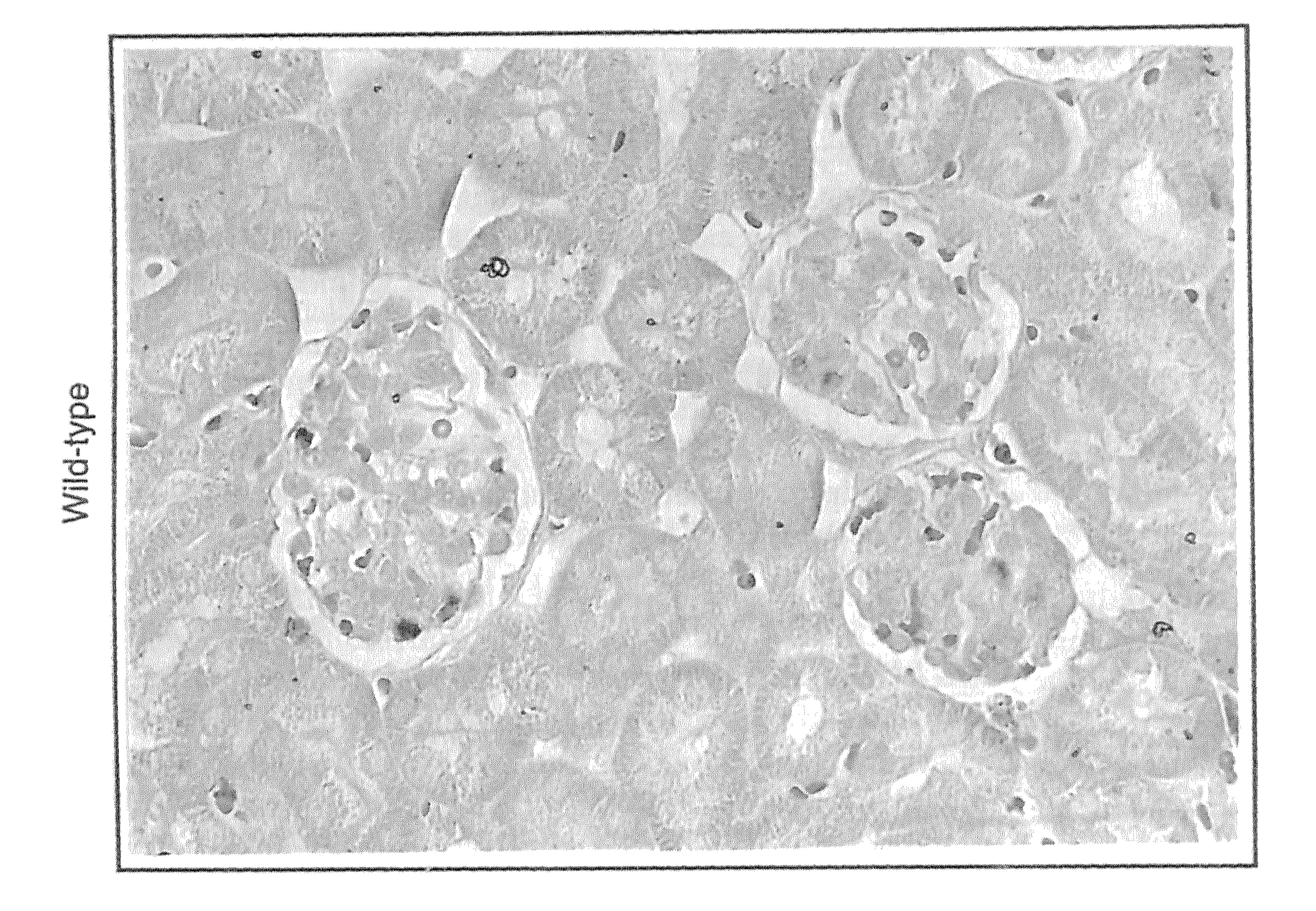


Fig. 1. Glomendar PMN accumulation. At indicated times after injection anti-GBM serum, kidneys were harvested and the number of PMNs per glomerular cross-section were determined in esterase stained sections. (A) A representative glomerular cross-section from a wild-type and mutant mouse two hours after induction of nephritis. The number of neutrophils (stained blue) per glomerular cross section in the mutant was higher than in the wild-type. Magnification is ×100. (B) Determination of glomerular neutrophil influx at different time points. Symbols are (23) wild-type; (2) mutant. Control animals (0 hr) received no antibody. For each animal, glomerular PMN counts in at least 150 glomeruli/two kidney sections were made. Data are presented as mean + sew. PMN levels were significantly elevated over baseline within 0.5 hours after antibody injection. Pselectin-deficient animals had approximately two fold more glomerular PMNs than wildtype animals at 0.5 hours, one hour, two hours and eight hours. *P < 0.005, #P <0.05. N = 7 for wild-type and for mutant mice for 0 hours. Similarly, N = 12 for 0.5 hours, N=15 for one hour and two hours, N=8 for eight hours and N = 4 for 24 hours.

declined significantly towards control values by 24 hours. Significantly higher numbers of PMN were observed in the glomeruli of P-selectin-deficient mice compared to wild-type animals (Fig. 1A). This difference was most striking at eight hours, when a 2.6-fold higher PMN influx was observed in mutant animals (Fig. 1B). Glomerular PMN counts in kidneys of untreated mutant and wild-type animals were not statistically different. The higher PMN influx in the P-selectin-deficient mice was associated with markedly higher albuminuria compared to wild-type animals (Fig. 2). There was no evidence of glomerular necrosis, thrombosis or significant tubulointerstitial PMN infiltrate at the time points examined in this model.

Anti-GBM antibody deposition was assessed in P-selectin-deficient and wild-type mice to determine whether the observed

differences in PMN influx could be explained by differences in glomerular immune complex formation. Qualitative analysis of glomerular immunoglobulin by indirect immunofluorescence of nephritic kidneys revealed comparable immunoreactivity in wild type and mutant mice. We also explored the possibility that the increased basal PMN counts in circulating blood of P-selectin-deficient mice (previously characterized to have 2.4 fold more circulating PMNs than wild-type animals) [5] might account for the increased PMN glomerular infiltrate in acute GN. White blood cell analysis was performed on the peripheral blood collected from wild-type and mutant mice at different times after induction of nephritis. Linear regression was performed of PMN/glomerular cross-section on circulating PMNs for each animal. PMN/ml of blood was not a significant predictor of PMN/

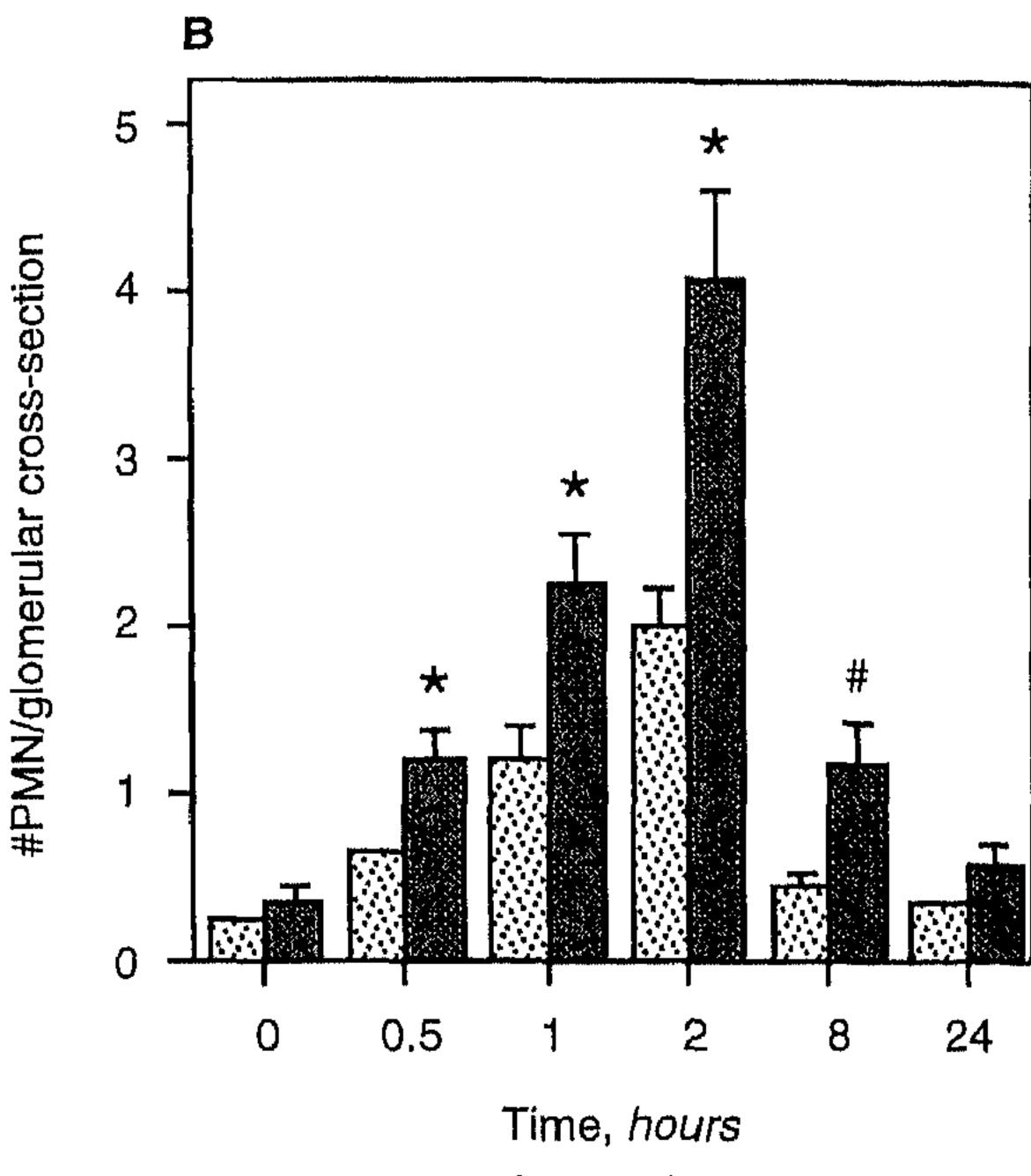


Fig. 1. Continued.

glomerular cross section in P-selectin-deficient mice with an R² of 0.00008. In contrast, a similar regression for wild-type mice predicted that PMN/ml of blood is a significant, albeit moderate, predictor of PMN/glomerulus; an R² of 0.38 suggesting that 38% of the variance in PMN recruitment could be explained by the variance in PMN/ml of blood. The R² for both wild-type and mutant animals was 0.069. Although the explanation for the differences in PMN correlation between wild-type and P-selectin-deficient mice is not clear, the results of the linear regression on P-selectin-deficient mice suggest that the increased PMNs in the glomerulus in mutant mice is probably not due to increased circulating neutrophils in these animals.

Generation of lipoxygenase-derived eicosanoids in experimental GN

We monitored renal LXA₄ levels in homogenates of nephritic kidneys from wild-type and P-selectin-deficient mice by ELISA to explore the possibility that the more prominent PMN infiltration in the latter group may be due, at least in part, to less efficient transcellular LXA₄ generation. Renal LXA₄ levels in P-selectin-deficient animals were approximately 60% of those obtained in wild-type animals at 0.5 and one hour after induction of nephritis (Table 1).

LXA₄ is generated by PMNs alone but the amount of LXA₄ generated per PMN is increased *in vitro* in the presence of platelets. For an index of the efficiency of LXA₄ generation by the leukocytes in the present *in vivo* study, we used a LXA₄ score which is calculated by dividing the levels of renal LXA₄ by the number of PMNs per glomerular cross section. The lipoxin A₄ score was approximately three- and twofold higher in the wild-type mice compared to P-selectin-deficient mice for the 0.5 hours and one hour time points, respectively.

In contrast to its influence on transcellular LXA₄ generation in acute GN, P-selectin deficiency did not diminish the generation of leukotriene B₄ (LTB₄) (Table 1). LTB₄ is a pro-inflammatory

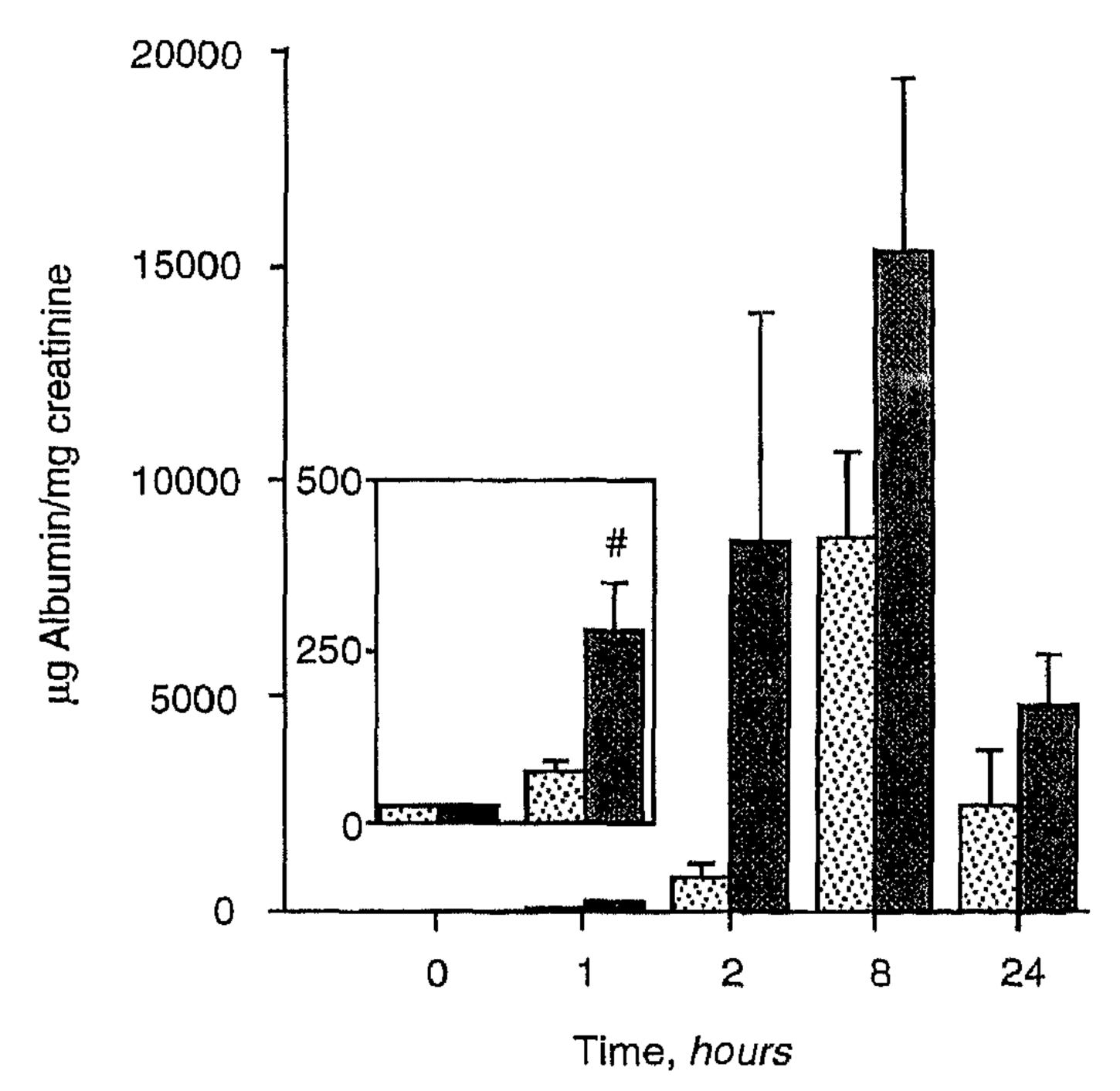


Fig. 2. Urinary albumin excretion. Symbols are: (121) wild-type; (122) mutant. Urine was collected over the duration of the experimental time period. Albumin and creatinine levels were determined by ELISA. Data are presented as mean \pm SEM. #P < 0.05. Albuminuria was significantly higher than baseline one hour after induction of nephritis, and peaked at eight hours. P-selectin-deficient mice had markedly higher albuminuria than wild-type counterparts at one, two and eight hours after induction of nephritis. Due to large variability between animals this was significant only at the one hour timepoint. The large SEM at two hours is due principally to the very high proteinuria (81,560 μ g ablumin/mg creatinine) obtained for one of the experimental mutant animals. Including this animal increases the average proteinuria for mutant mice such that the difference in albuminuria between wild-type and P-selectin-deficient mice is 10-fold and not statistically significant. Excluding this animal gives a 4-fold difference, which is statistically significant (P < 0.05). Numbers of animals are the same as for those listed in Figure 1, except for 0 hours urine where N = 48.

Table 1. Renal lipoxin A₄ and leukotriene B₄ levels

	Wild-type	P-selectin-deficient
LXA ₄ ng/g kidney		
0 hr	$4.7 \pm 0.9 (7)$	$3.3 \pm 0.4 (7)$
0.5 hr	$19.4 \pm 2.4 (8)$	$12.6 \pm 1.5 (8)^{b}$
1.0 hr	$15.8 \pm 1.7 (11)$	$9.2 \pm 1.0 (11)^{a}$
2.0 hr	$16.8 \pm 2.1 (7)$	$13.9 \pm 3.2 (7)$
LXA ₄ score		
0 hr	20.6 ± 3.1	14.0 ± 4.3
0.5 hr	35.6 ± 4.4	$10.8 \pm 0.7^{\rm b}$
1.0 hr	15.3 ± 2.1	$6.8 \pm 1.7^{\rm n}$
2.0 hr	10.1 ± 1.8	7.6 ± 3.1
LTB ₄ ng/g kidney		
0 hr	16.8 ± 3.1	11.2 ± 1.8
0.5 hr	398.6 ± 87.2	349.5 ± 69.5
1.0 hr	397.1 ± 81.2	352.9 ± 71.4
2.0 hr	495.5 ± 151.9	315.1 ± 68.5

Results indicate mean ± SEM. Number of animals for each set of LXA₄ values is given in parentheses and is the same for LXA₄ score and LTB₄ values.

lipoxygenase-derived eicosanoid, that is generated from LTA₄ within PMNs and does not require transcellular biosynthesis with platelets [8]. This result suggested that the decrease in LXA₄

a P < 0.005, b P < 0.05.

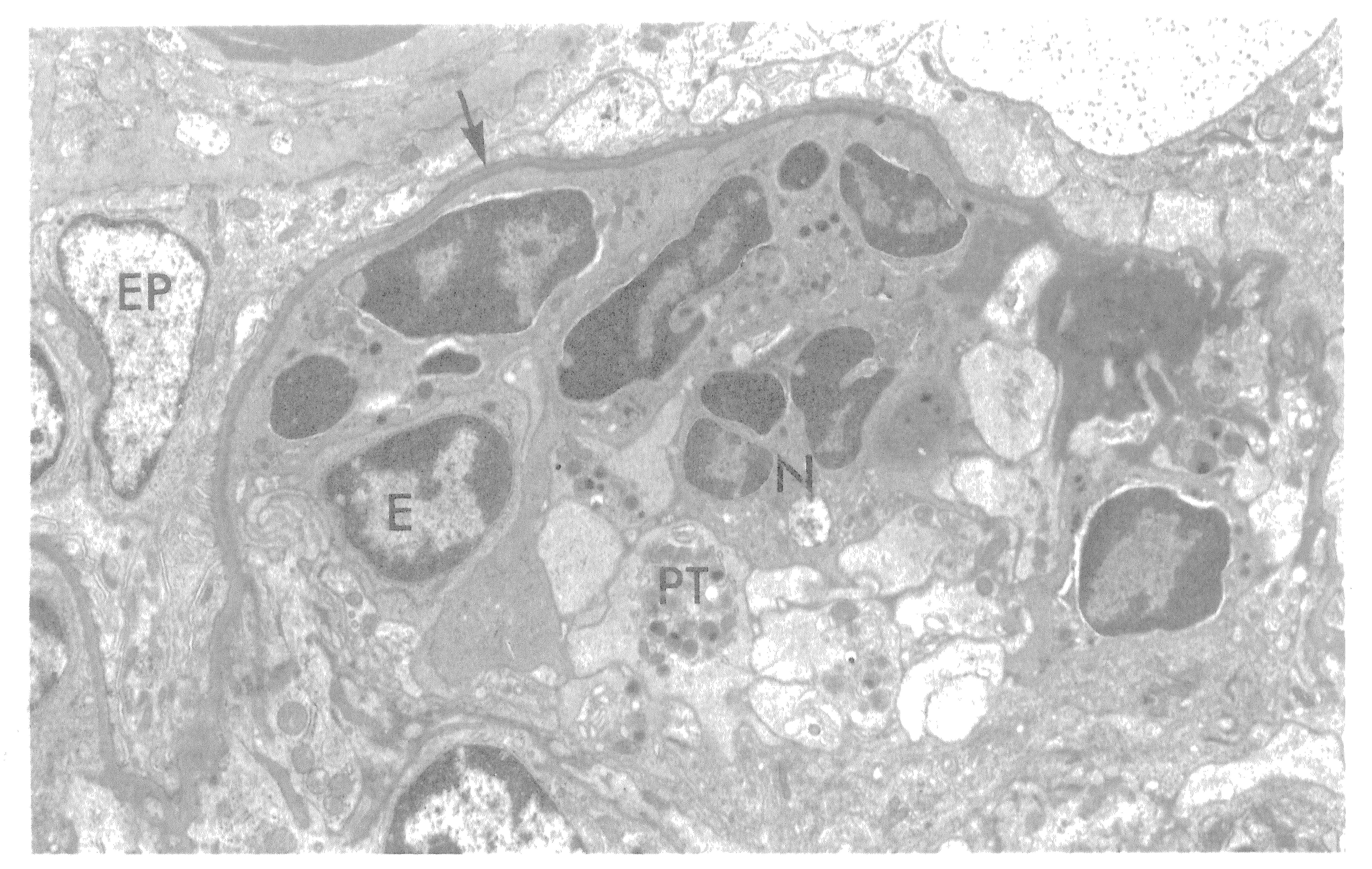


Fig. 3. Electron micrograph of nephritic kidney of P-selectin-deficient mouse two hours after infusion with anti-GBM antibody. The micrograph shows a glomerular capillary loop with several platelets (PT) in close proximity to intraglomerular PMNs (N). There are areas of partially denuded fenestrated endothelium (E) with inflammatory cells in close contact with the basement membrane (arrow), and the visible epithelial cells (EP) reveal prominent effacement (fusion) of foot processes. Magnification is ×7,250.

levels was not secondary to decreased availability of PMN-derived LTA₄ for conversion by platelets to LXA₄.

Role of P-selectin on platelets in renal LXA4 generation, PMN influx and proteinuria

An ultrastructural analysis of glomeruli of P-selectin-deficient mice two hours after induction of nephritis revealed the coexistence of a number of PMNs and platelets in the glomerular capillary lumen (Fig. 3). This suggested that P-selectin deficiency did not inhibit localization of platelets to the inflammatory site. To determine whether P-selectin-containing platelets could correct the deficiency in LXA, production in P-selectin deficient mice, we injected approximately 1.1 to 1.7 × 10⁸ wild-type platelets into mutant and wild-type mice, 15 to 20 minutes prior to injection of anti-GBM antibody. P-selectin-deficient mice injected with wild-type platelets had a statistically significant increase in LXA, levels at one hour compared to P-selectin-deficient mice which received no platelets (P < 0.005). More importantly, mutant mice (+platelets) had lipoxin A, levels that were indistinguishable from LXA, levels obtained for wild-type mice (+platelets) (Table 2). In contrast to its effect on LXA, generation, platelet transfusion did not influence LTB, formation in mutant mice when compared to wild-type mice. Interestingly, LTB₄ levels were lower in both platelet-transfused mutant and wild-type mice than in non-transfused animals, perhaps reflecting increased

Table 2. Renal lipoxin A_4 and leukotriene B_4 levels following platelet infusion

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		92 mars 10 (11)
1.0 hr - platelets	17.0 - 2.1 (7)	17.7 - 3.0 (7)
LXA, SCOTC	**	
1.0 hr	I may an	6 8 major 1 7 a
1.0 hr + platelets	100 - 13	100 min 20
LTB ₄ ng/g kidney		
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1.0 hr - platelets	The second secon	man of the second of the secon

Results indicate mean ± SEM. (+) platelets indicates that animals received an intravenous injection of wild-type platelets 15 minutes prior to injection of anti-GBM antibody. Number of animals for each set of LXA₄ values is given in parentheses and is the same for LXA₄ score and LTB₄ values.

platelet recruitment to glomeruli and redirection of LTA₄, the key intermediate for LTB₄ production, for production of LXA₄ (Table 2). Together, these results provide further evidence that platelet-PMN adhesion through P-selectin enhances transcellular LXA₄ generation by platelet-PMN biosynthetic pathways in acute GN.

a P < 0.005

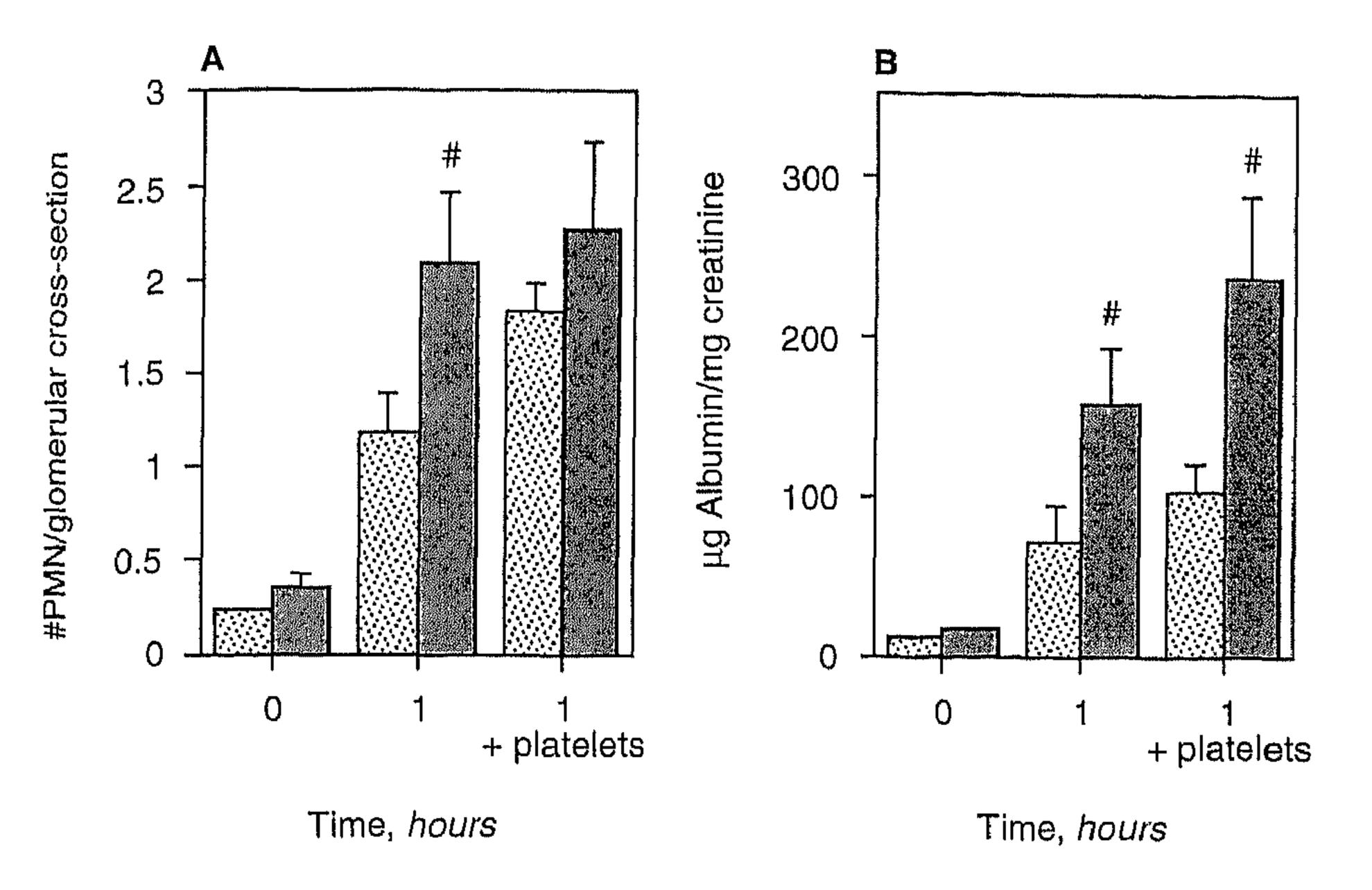


Fig. 4. Glomerular PMN accumulation and urinary albumin excretion before and after platelet injection. Data are presented as mean ± SEM. #P < 0.05. Similar to the results shown in Figure 1, glomerular PMN influx and albuminuria in P-selectin-deficient mice were significantly higher than in wild-type animals at one hour. After injection of platelets, glomerular PMN accumulation was no longer significantly different in wild-type and Pselectin-deficient animals (P < 0.39). However, urinary albumin excretion remained significantly higher in P-selectin-deficient mice receiving platelets compared to wild-type animals receiving platelets. Symbols are: (12) wild-type; (🖾) mutant.

A comparison of corresponding glomerular PMN accumulation after platelet infusion in wild-type and P-selectin-deficient mice shows that the difference in glomerular PMN accumulation between these two groups of animals had narrowed so that they were no longer significantly different (Fig. 4A). However, albuminuria remained significantly higher in P-selectin-deficient mice in comparison to wild-type mice (Fig. 4B).

Discussion

There are two major findings in this study. The first is that P-selectin deficiency leads to significantly higher glomerular PMN influx and markedly increased albuminuria, compared to wildtype animals. The findings are surprising in that three sets of previous studies suggest an important role for P-selectin in early PMN influx in vivo. First, antibodies to P-selectin inhibit PMN influx and PMN-mediated injury in a wide variety of inflammatory models from ischemia-reperfusion to complement-induced lung injury [2]. Second, P-selectin-deficient mice have delayed leukocyte influx in thioglycollate-induced peritoneal inflammation [5] and in delayed hypersensitivity reaction [18]. Third, a polyclonal P-selectin antibody has been shown to decrease the PMN influx and albuminuria in a similar murine model of anti-GBM nephritis, at the same time periods [19]. The explanation for the increased neutrophil influx and proteinuria in P-selectin-deficient animals is not firmly established by these studies, but we considered a number of mechanisms. We found no evidence that there was increased binding of anti-GBM antibody in P-selectin-deficient mice, and thus this possibility was excluded. A second explanation may lie in the basal blood neutrophilia, which is seen in the majority of P-selectin-deficient mice [5]. However, we found no consistent correlation between the circulating neutrophil count and the number of neutrophils per glomerular section; linear regression analysis showed that PMN/ml of blood was not a significant predictor of PMN/glomerular section in P-selectindeficient mice, and the linear regression of the entire groupdeficient and wild-type-showed that PMN levels did not predict glomerular neutrophil counts. Nevertheless, this mechanism cannot be entirely excluded, since the neutrophil blood counts were performed at only one time period, which may not have been

representative of leukocyte counts during the entire period of observation. A resolution of the role of leukocytosis must await ongoing studies using mice deficient in other adhesion molecules. However, even if leukocytosis were the cause of the increased influx and proteinuria, this would be the first demonstration of such an effect in acute nephritis.

The second principal finding of this study is the decreased lipoxin A₄ generation in kidneys of P-selectin-deficient mice. Papayianni et al [1] have recently provided evidence that LXA₄ is generated within minutes after induction of immune complex-mediated GN in rats via platelet-PMN transcellular pathways, that P-selectin-mediated platelet PMN adhesion promotes the LXA₄ synthetic process *in vitro*, and that P-selectin antibody attenuates lipoxin A4 generation *in vivo*. These authors also showed that LXA₄ inhibits leukotriene induced neutrophil adhesion to vascular endothelium by P-selectin and integrin-dependent mechanisms [10]. Taken together, these observations raise the possibility that the more prominent PMN influx and glomerular injury observed in P-selectin-deficient mice in our study may have been due, at least in part, to diminished transcellular biosynthesis of lipoxin A4 via platelet-PMN routes.

Indeed, LXA₄ levels in renal tissue of nephritic kidneys of P-selectin-deficient mice was 60% of wild-type levels whereas LTB₄, which is generated from LTA₄ by PMNs alone was unaffected. Whereas a contribution from cross reacting substances cannot be excluded, three lines of evidence suggest that LXA4 was the major immunoreactive substance detected by ELISA in our study and points to the usefulness of this assay for monitoring levels of this product in the murine system. First, the ELISA has a threshold of sensitivity of 90.8 fmol LXA₄/ml and cross reactivity of this antibody for leukotrienes and monohydroxyeiconsatetraenoic acids is < 0.1% [17]. These characteristics were highlighted in our study by the divergence among renal levels of LXA4 and LTB₄, the latter being the major 5-lipoxygenase product produced during acute glomerular inflammation. Second, the lipoxin biology defined by this ELISA during glomerulonephritis in vivo closely parallels that observed in activated multicell systems in vitro. The nephrotoxic serum used in our model induces glomerular inflammation that is remarkable for the abundance of both platelets and neutrophils within the glomerular capillary lumen, platelet-neutrophil transcellular pathways being the most abundant source of LXA₄ in vitro. Attenuation of platelet-neutrophil adhesion in vivo by gene knockout of P-selectin blunted LXA₄ generation, an effect also seen during platelet-neutrophil interactions in vitro in the presence of anti-P-selectin monoclonal anti-body [1]. Third, a recent study by Carey et al [20] demonstrates the generation of immunoreactive epi-lipoxins in the kidneys of aspirin-treated rats during glomerulonephritis, presumably as a result of neutrophil-endothelial interactions known to occur in the presence of inhibitors of cyclooxygenase II in vitro.

The decrease in LXA4 generation in P-selectin-deficient animals was not a consequence of a lack of platelet recruitment since both platelets and PMN were observed in intimate context within the glomerular capillary lumen at the height of disease in our model. Our results suggest that P-selectin is an important modulator of transcellular LXA4 generation in experimental complement-independent anti-GBM nephritis. The efficiency of LXA₄ production, expressed as LXA₄ generated per PMN, was also twoto threefold lower in P-selectin-deficient mice than in their wild-type counterparts. Whereas this trend was observed for all time points studied, it proved more striking at 30 minutes and one hour, by which time platelet infiltration is prominent [16, 21]. These observations suggest the importance of PMN-platelet interactions as an initial source of LXA4 during acute inflammation. It is likely that cytokine-activated glomerular endothelial, mesangial and epithelial cells, and/or monocytes contribute to transcellular LXA₄ generation following induction of their 12/15-lipoxygenase activity during the evolution of the inflammatory response [22, 23]. PMN interactions with these cells may account for the lesser role of P-selectin in transcellular LXA₄ generation at later times.

The injection of wild-type platelets into P-selectin-deficient mice amplified LXA₄ levels to those of wild-type mice in acute GN and improved the efficiency of LXA4 generation. Platelet infusion studies also provided evidence that, although additional pathways for lipoxin biosynthesis are still being defined, platelet P-selectin is an important regulator of transcellular LXA₄ generation during the initial stages of acute GN. Platelet infusion also approximated glomerular PMN infiltrate in the two groups of mice. It should be noted, however, that glomerular PMN influx, while comparable, was actually slightly higher in both P-selectindeficient and wild-type animals following infusion of wild-type platelets than in non-transfused animals. This is not unexpected since platelets are known to play a pro-inflammatory role in PMN recruitment and renal injury in several models of acute immune complex-mediated GN [1, 21, 24]. The complex changes in glomerular PMN infiltration observed following platelet infusion reflect the combined, and potentially opposing, effects of platelets on PMN recruitment and activation, transcellular eicosanoid generation and possibly other key multicellular inflammatory events. The complexity of these in vivo responses was further highlighted by the observation that platelet transfusion did not alter the differential protein excretion between P-selectin-deficient and wild-type mice, despite approximating renal LXA₄ levels and PMN infiltrates.

Although the net effect of P-selectin deficiency in this model does not lead to decreased or delayed leukocyte influx, as predicted from antibody inhibition studies, the results do not argue against a role for endothelial P-selectin in leukocyte recruit-

ment, since the inflammatory effects of LXA4 deficiency may have masked the anti-adhesive effects of endothelial P-selectin deficiency. Indeed, one explanation for the difference between our results and those of Tipping et al [19], who did inhibit neutrophil influx and albuminuria with a polyclonal P-selectin antibody, is that their administered dose of P-selectin antibody was not sufficient to block P-selectin on platelets that is present at 13-fold higher levels than on endothelial cells [25]. Other possibilities for the discrepancy are that the polyclonal antibody may have physically interfered with the function of other leukocyte adhesion molecules while bound to P-selectin on endothelium. It may have cross-reacted with other adhesion molecules or may have influenced the PMN function or adhesion to endothelium or platelets via mechanisms unrelated to reactivity with P-selectin. In our case we cannot exclude the possibility that deletion of the P-selectin gene induced up-regulation of the expression of other genes that promote PMN recruitment in our nephritis model.

In conclusion, we have demonstrated that P-selectin deficiency does not inhibit leukocyte influx in a model of acute anti-GBM nephritis in the mouse; on the contrary, it leads to increased neutrophil PMN recruitment and albuminuria compared to wild-type mice. We also show that P-selectin deficiency leads to decreased LXA₄ generation in this model by interfering with platelet-neutrophil interactions. Although our experiments do not link these two P-selectin dependent effects, it is possible that the decreased LXA₄ generation may contribute to the increased neutrophil events. These studies highlight the complexity of the interactions between platelets, endothelial cells and neutrophils in inflammation and the need to consider the effects on each set of interactions when interpreting studies in which molecules that mediate the inflammatory response are ablated.

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