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J Clin Invest. 1994;**94**(3):928-936. <https://doi.org/10.1172/JCI117459>.

Research Article

The metabolic and functional alterations which occur during the acute phase of nephrotoxic nephritis (NTN) in rats, a model of immune-mediated glomerulonephritis, result from a cooperative interaction between PMNs and platelets (PLTs). In consequence, we hypothesized that fibrinogen (Fg) might play a critical role in this process and, accordingly, we found that defibrination of animals decreased both the acute phase proteinuria in NTN (approximately 70%) as well as the influx of PLTs and PMNs into the glomerulus (approximately 40-50%). In contrast, blockade of the PLT Fg receptor, alpha IIb beta 3, with the RGD peptidomimetic SC-49992 decreased proteinuria (approximately 90%) without substantially altering the influx of PMNs or PLTs. Immunocytochemistry showed a marked increase in beta 3 integrin expression in inflamed glomeruli which was prevented either by PMN or PLT depletion before disease induction. FACS and immunocytochemical analysis of glomerular cell dissociates demonstrated that beta 3 integrin expression was predominantly on intraglomerular PLTs. In vitro, activated PLTs stimulated the PMN respiratory burst, an interaction which could be inhibited by Fg receptor blockade. In sum, acute NTN is accompanied by a marked increase in glomerular beta 3 integrin expression predominantly due to the influx of PLTs which localize to the glomerulus in a PMN-dependent fashion. Fg appears to serve a major role as a coactivating stimulus for PLT-PMNs in situ via [...]

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Fibrinogen Mediates Platelet–Polymorphonuclear Leukocyte Cooperation during Immune-Complex Glomerulonephritis in Rats

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Abstract

The metabolic and functional alterations which occur during the acute phase of nephrotoxic nephritis (NTN) in rats, a model of immune-mediated glomerulonephritis, result from a cooperative interaction between PMNs and platelets (PLTs). In consequence, we hypothesized that fibrinogen (Fg) might play a critical role in this process and, accordingly, we found that defibrination of animals decreased both the acute phase proteinuria in NTN (~ 70%) as well as the influx of PLTs and PMNs into the glomerulus (~ 40–50%). In contrast, blockade of the PLT Fg receptor, $\alpha_{IIb}\beta_3$, with the RGD peptidomimetic SC-49992 decreased proteinuria (~ 90%) without substantially altering the influx of PMNs or PLTs. Immunocytochemistry showed a marked increase in β_3 integrin expression in inflamed glomeruli which was prevented either by PMN or PLT depletion before disease induction. FACS® and immunocytochemical analysis of glomerular cell dissociates demonstrated that β_3 integrin expression was predominantly on intraglomerular PLTs. In vitro, activated PLTs stimulated the PMN respiratory burst, an interaction which could be inhibited by Fg receptor blockade. In sum, acute NTN is accompanied by a marked increase in glomerular β_3 integrin expression predominantly due to the influx of PLTs which localize to the glomerulus in a PMN-dependent fashion. Fg appears to serve a major role as a coactivating stimulus for PLT–PMNs in situ via $\alpha_{IIb}\beta_3$, potentially mediating the PMN respiratory burst which contributes to proteinuria. Fg may also play a subsidiary role in PMN/PLT comigration. (*J. Clin. Invest.* 1994. 94:928–936.) Key words: glomerulonephritis • fibrinogen • β_3 integrin • neutrophil • platelet

Introduction

Nephrotoxic nephritis (NTN)¹ in rats serves as a model system for immune-mediated glomerulonephritis in man (1, 2). Studies

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Received for publication 14 January 1994 and in revised form 6 May 1994.

1. Abbreviations used in this paper: CD45, leukocyte common antigen; Fg, fibrinogen; HRP, horseradish peroxidase; NTN, nephrotoxic nephritis; PLT, platelet.

J. Clin. Invest.

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0021-9738/94/09/0928/09 \$2.00

Volume 94, September 1994, 928–936

into the pathogenesis of this disease model show that leukocytes are critical effectors of the glomerular damage and dysfunction in this disease model and the accompanying alterations in glomerular biochemistry (1, 2). Because of the central role played by leukocytes in this form of experimental glomerulonephritis, the mechanisms by which these cells enter glomeruli in the context of inflammation, become activated, and induce glomerular dysfunction are of topical concern.

With respect to PMNs (which mediate the acute phase of NTN) it has become clear that their emigration into glomeruli is a complex function of the activation of complement, the production of local cytokines, the upregulation of vascular adhesion molecules, and the expression of β_2 integrins on the PMNs (3–5). In addition, in recent work by our laboratory, it appears that platelets (PLTs), as well, are essential for the efficient influx of PMNs into the glomerulus and contribute to the acute glomerular injury (6). Specifically, we have found that PLTs are essential for the influx of PMNs into the glomerulus, the upregulation in glomerular leukotriene B₄ production, and the evolution of proteinuria (6). Conversely, PMNs are essential for the influx of PLTs into the glomerulus as well as the upregulation in glomerular thromboxane production (6) and are clearly a major effector for producing proteinuria (7).

PLT–PMN interactions appear to be a common part of the acute inflammatory response and may be mediated via a myriad of different molecular mechanisms (8, 9). In an effort to broach this general issue in the context of NTN, we focused specifically on fibrinogen (Fg). We hypothesized that Fg would play a critical role in the interaction of PLTs and PMNs in inflamed glomeruli based on prior in vivo data suggesting that defibrination/fibrinolysis could ameliorate experimental glomerulonephritis (although not invariably) (10, 11) and on in vitro evidence that Fg could serve to activate PLTs and PMNs as well as play a role in their migration via effects on adherence and chemotaxis (12, 13). Underscoring the potentially important role of Fg in inflammation are data which show that Fg alone (even in the absence of complement or immunoglobulin) is sufficient to render inert materials proinflammatory (14).

By comparing and contrasting defibrination with Fg receptor blockade in vivo, by probing for β_3 integrin expression in vivo using immunocytochemical techniques, and by examining PLT–PMN interactions in vitro, we were able to establish a central role for Fg and its receptor, $\alpha_{IIb}\beta_3$, in the development of the acute phase of NTN. Moreover, the data would appear to resolve the participation of Fg into two separate components: PLT–PMN coactivation in situ and PLT–PMN comigration into glomeruli.

Methods

Reagents and materials. Lewis rats were obtained from Charles River Breeding Laboratories (Wilmington, MA). Collagenase type I, trypsin

type III, EDTA, diaminobenzidine, FITC-conjugated sheep anti-mouse IgG Fab fragments, thrombin, cytochrome *c*, and Ancrod were purchased from Sigma Chemical Co. (St. Louis, MO). PMA was obtained from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Glycogen was obtained from Fisher Scientific (Fair Lawn, NJ). SC-49992, a peptidomimetic of the Fg binding sequence RGD, was provided by Searle Pharmaceuticals (Skokie, IL). Hematoxylin was obtained through EM Diagnostic Systems (Gibbstown, NJ). FITC-conjugated goat anti-rat Fg, FITC-conjugated affinity-purified goat anti-rabbit IgG, and FITC-conjugated goat anti-rat C3 were purchased from Cappel Laboratories (West Chester, PA). Monoclonal anti-rat leukocyte common antigen (CD45) was purchased from Chemicon International, Inc. (Temecula, CA). Horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG was purchased from Bio-Rad Laboratories (Richmond, CA). Rabbit anti-rat PMN polyclonal antiserum was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY).

Monoclonal antibody to β_3 integrin was raised and characterized as previously described (F11) (15). Rabbit anti-rat PLT polyclonal antiserum was raised and characterized also as described previously (6). For visualizing the PLTs in glomerular cell preparations, this antibody was purified using protein A-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) and directly labeled with HRP using a kit from Zymed Laboratories, Inc. (South San Francisco, CA).

Induction of NTN and its pharmacologic/immunologic manipulation. The preparation of nephrotoxic serum has been detailed previously (6). This antiserum contains no detectable endotoxin (< 0.1 U/ml). To induce nephritis, rats were given 5 ml/kg intravenously (i.v.). Urine was collected using a metabolic cage, and proteinuria was measured by the Bradford assay (Bio-Rad Laboratories). Glomeruli and renal tissue were harvested 3 or 24 h after disease induction for the analyses below. Glomeruli were harvested from kidneys perfused free of blood.

Various pharmacologic/immunologic manipulations were performed as follows: to defibrinate animals, Ancrod 50 U/kg was injected subcutaneously thrice daily for 5 d before disease induction. Prolonged treatment with Ancrod is necessary to deplete the internal pool of Fg within PLTs (16). PLT depletion was induced before induction of disease by the injection of the aforementioned anti-PLT antiserum 10 ml/kg which decreases circulating PLT counts to undetectable levels but does not affect circulating leukocyte/PMN counts (reference 6 and data not shown). PMN depletion was generated by injection of anti-PMN antiserum 5 ml/kg. This protocol decreased circulating PMNs by $> 90\%$ but did not affect circulating PLT counts (data not shown).

To effect Fg receptor blockade, animals were given SC-49992, an RGD peptidomimetic (17, 18). Although RGD peptides and their mimetics are not generally effective in inhibiting rat PLT aggregation *in vitro*, some RGD peptide analogues have been shown to prevent collagen-induced thrombocytopenia in rats *in vivo* (19, 20). We therefore used a potent mimetic of the RGDF tetrapeptide, SC49992, which has been demonstrated to inhibit human and dog PLT aggregation (17, 18) and to prevent collagen-induced thrombocytopenia in the rat (ED₅₀ 53 $\mu\text{g}/\text{kg}$).

We used a variant of the above protocol for induction of NTN because the short half-life of SC-49992 necessitated i.v. infusion. In these experiments, an intraarterial cannula was placed in the femoral artery under anesthesia to allow infusion of SC-49992. Animals were then allowed to recover, during which time they were infused with saline 2 ml/h to help maintain urine flow. After a 2-h period of equilibration, animals were injected with SC-49992 1 mg/kg or an equivalent amount of vehicle (saline) followed by nephrotoxic serum 5 ml/kg. For the next 3 h, animals were either given an infusion of SC-49992 80 $\mu\text{g}/\text{kg}$ per minute in saline or an equivalent volume of saline, at 2 ml/h, and urine was collected. At the end of 3 h, animals were killed as above to obtain glomeruli or tissue for the following analyses.

Glomerular isolation, cell dissociation, and immunostaining. Glomeruli were isolated from saline-perfused kidneys using a sieving protocol described previously which yields a preparation of glomeruli 80–90% pure (6). The glomeruli were then dispersed into single cell preparations by a previously published enzymatic dissociation protocol (6).

This protocol yields a single cell suspension of mesangial cells and leukocytes. Occasional endothelial cells are present; however, epithelial cells are largely lost. Cell counts were performed after dissociation to obtain total cell yields and to assess viability (uniformly $> 90\%$ by trypan blue exclusion).

Glomerular leukocytes were quantified in these cell dissociates by immunocytochemistry. Aliquots of cell suspensions ($\sim 30,000$ cells) were pelleted onto slides by cytocentrifuge (Shandon Inc., Pittsburgh, PA). The cells were fixed with methanol/acetone (50:50), and leukocytes were labeled with an anti-CD45 antibody as previously detailed (6). Hematoxylin was used as a counterstain. The positive labeled cells were quantified as a percentage of the total by light microscopy and categorized by nuclear morphology as to lineage. Leukocyte counts per glomerulus were then calculated by multiplying leukocyte percentages (or subset fractions) by the total glomerular cell number. This technique correlates well with other methods of leukocyte quantification, such as *in situ* staining.

A similar protocol was used to assess the presence of PLTs in the glomerular cell dissociates using the aforementioned HRP-labeled anti-PLT polyclonal antibody as the detecting reagent (6). PLTs were identified by positive staining as well as appropriate morphology.

For FACS[®] analysis, 1×10^6 glomerular cells were incubated with anti-rat CD45 or anti-rat β_3 integrin monoclonal antibodies for 45 min in PBS with 10% goat serum at 4°C, washed two times with PBS, and finally incubated with sheep anti-mouse IgG F(ab')-FITC for 45 min at 4°C. Cells were again washed two times, and the samples were analyzed on a FACScan[®] (Becton Dickinson, Mountain View, CA).

Glomerular immunohistology. Glomerular depositions of rabbit IgG, rat C3, and rat Fg were determined by direct immunofluorescence on fresh frozen tissue using the aforementioned antibodies and previously published protocols (21). Glomerular expression of CD45 or β_3 integrin was visualized in fresh frozen tissue using the appropriate primary antibodies (see above, 1:100 dilution in PBS with 10% goat serum) along with an avidin-biotin-HRP kit from Vector Labs Inc. (Burlingame, CA). The secondary antibody in the kit was a biotinylated horse anti-mouse IgG. Staining used diaminobenzidine as the HRP substrate and hematoxylin as the counterstain.

PLT-PMN interactions *in vitro*. PMNs were isolated by peritoneal lavage from rats injected with 7 ml of 1% glycogen intraperitoneally 4 h previously (preparations $> 95\%$ pure). PLTs were isolated from blood diluted 1:2 with heparinized saline by centrifugation at 800 g and washed with HBSS before use. Incubations for the respiratory burst were performed in a volume of 0.4 ml and contained PMNs (2×10^6) without or with PLTs (2×10^8). Cytochrome *c* (1 mg/ml) was included in the incubation as an indicator of superoxide production. Incubations were stimulated with either PMA (1 μM) or thrombin (3 U/ml) for 30 min at 37°C. In experiments to examine the role of Fg receptor blockade, SC-49992 (1 mg/ml) or anti- β_3 monoclonal antibody (10 $\mu\text{g}/\text{ml}$) was preincubated with cells for 15 min at 37°C before stimulation. Superoxide production was measured by spectrophotometry (absorbance at 549 nm) and is expressed in relative units normalized for the agonist control (agonist stimulation = 100).

Statistical analysis. Statistical analysis was performed with the aid of STATA (Computing Resource Center, Los Angeles, CA). Values are expressed as mean \pm SEM. Each replicate represents the data from one animal. Two-way comparisons were made with a two-tailed Student's *t* test (unpaired or paired as appropriate). When comparisons were made between multiple groups, ANOVA was performed along with Bonferroni's correction for multiple comparisons. Significance levels are indicated in the text or figure legends.

Results

Defibrination and the evolution of NTN. To begin to dissect out the role of Fg in the development of the inflammatory lesion of NTN, rats were defibrinated with Ancrod for a period of 5 d before disease induction to deplete both circulating levels of

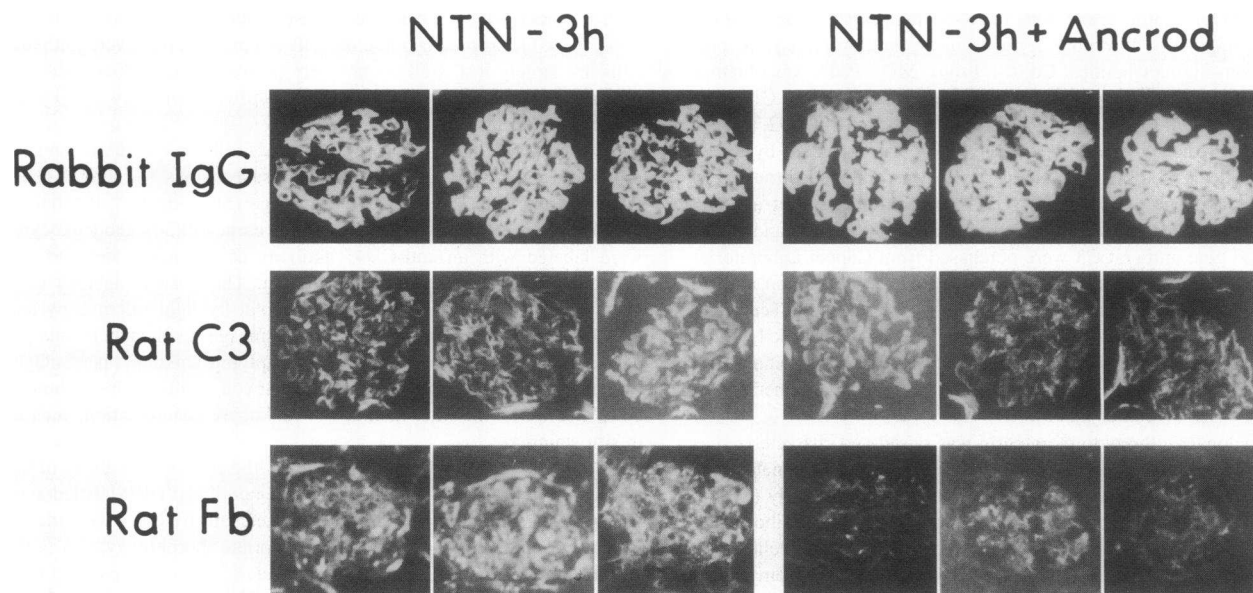


Figure 1. Effect of defibrination on the intraglomerular deposition of heterologous IgG and endogenous C3 and Fg during NTN. NTN was induced with rabbit polyclonal antibody to rat glomerular basement membrane (GBM) in unmanipulated and defibrinated animals. Deposition of rabbit IgG and rat C3 and Fg were detected by direct immunofluorescence on fresh frozen renal tissue obtained 3 h after disease induction. The figure presents representative glomeruli from a nephritic and a nephritic-defibrinated animal. No staining was evident for rabbit IgG, rat C3, or rat Fg in normal controls (not shown).

Fg as well as the reservoir of Fg within PLTs (16). To evaluate the efficacy of defibrination, we examined the effect of this protocol on the intraglomerular deposition of Fg after induction of NTN. As expected, defibrination markedly attenuated the deposition of Fg in the glomerulus during the acute phase of nephritis, although there was no substantial effect on the deposition of either rabbit IgG (nephrotoxic serum) or C3 (Fig. 1).

We concomitantly assessed the effect of Fg depletion on the proteinuria which accompanies NTN and which serves as an index of the invasion of inflammatory cells into the glomerulus and their activation (7). Animals given nephrotoxic serum exhibited an approximately 10-fold elevation in urine protein over the 24 h after disease induction compared with normal controls (Fig. 2). Defibrinated nephritic animals developed substantially less proteinuria than their nephritic counterparts (ap-

proximately a 70% decrease compared with nephritic animals, Fig. 2; Fg comprises < 5% of the plasma protein content).

Because of the strong correlation between glomerular leukocyte numbers and proteinuria in NTN (7), we next addressed whether the decrease in proteinuria which resulted from defibrination occurred *pari passu* with a decrease in the influx of leukocytes. As we have found in past studies, NTN was characterized by a marked increase in glomerular leukocytes at 3 and 24 h: PMNs predominated at the early time point but were replaced by macrophages at 24 h (Fig. 3). With Fg depletion there was a modest decline in glomerular leukocytes at both time points although the change was not statistically significant (approximately a 25–30% decrease, Fig. 3). In terms of leukocyte subpopulations, though, Fg depletion significantly diminished the influx of PMNs at 3 h (by ~ 50%).

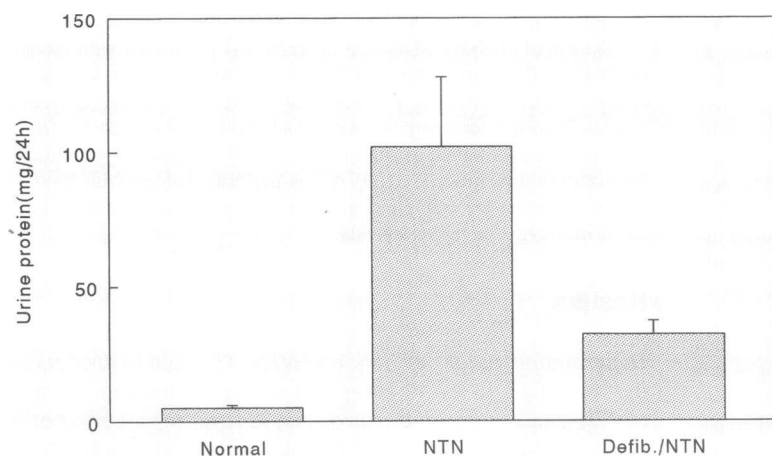


Figure 2. Effect of defibrination on proteinuria in NTN. NTN was induced with a rabbit polyclonal antibody to rat GBM in unmanipulated and defibrinated animals. Nondisease controls were also assessed. Urine was collected for 24 h after disease induction, and the protein content was calculated by a microspectrophotometric assay. $n = 3, 5,$ and 6 for the three groups shown (left to right: normal, nephritic, nephritic/defibrinated). The difference between nephritic and defibrinated-nephritic animals was significant ($P < 0.05$).

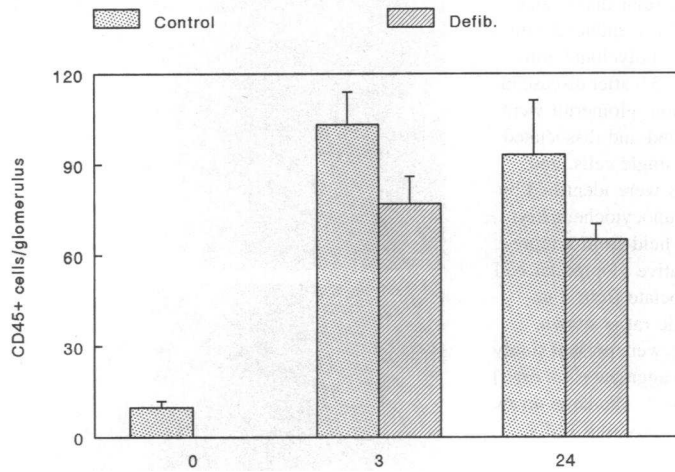
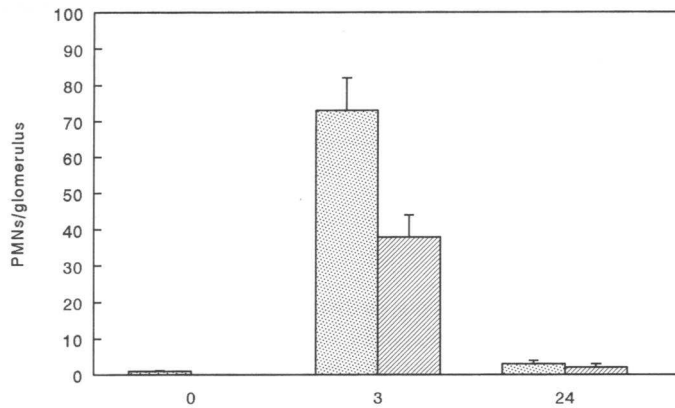
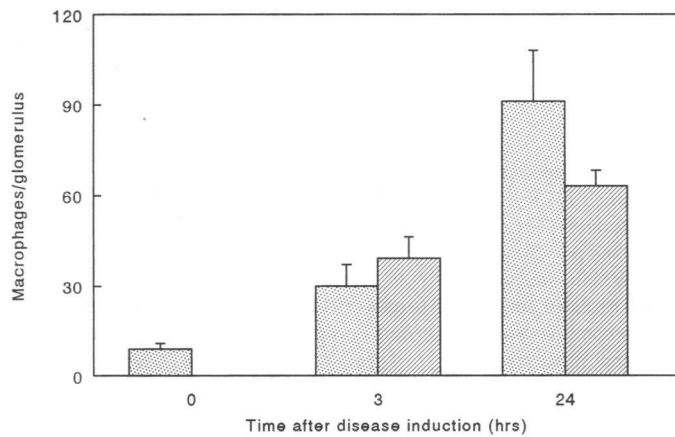
A**B****C**

Figure 3. Effect of defibrination on leukocyte influx into the glomeruli in NTN. NTN was induced with a rabbit polyclonal antibody to rat GBM in unmanipulated and defibrinated animals. Nondisease controls were also assessed. Glomeruli were isolated at 0, 3, or 24 h after disease induction and dissociated into single cells, and leukocytes were quantified and subtyped by immunocytochemical staining. $n = 3, 6, 7, 5,$ and 6 for the five groups shown (left to right: normals, nephritic at 3 h after disease induction, nephritic/defibrinated at 3 h after disease induction, nephritic at 24 h after disease induction, and nephritic/defibrinated at 24 h after disease induction). **A,** total leukocytes/glomerulus; **B,** PMNs/glomerulus. The difference between nephritic and defibrinated-nephritic animals for glomerular PMN counts at 3 h was significantly different ($P < 0.05$). **C,** macrophages/glomerulus.

Since a cooperative interaction between PLTs and PMNs is critical to the efficient influx of PMNs into the glomerulus (6), we additionally quantified the PLT content of glomerular cell

dissociates. As shown in Fig. 4, dissociates from nephritic glomeruli contained substantial numbers of PLTs, singly or in aggregates, both cell-associated and non-cell-associated. PLTs

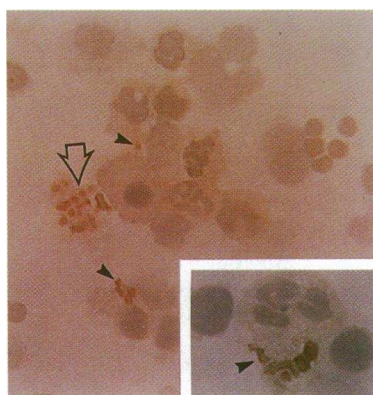


Figure 4. PLT content of glomerular dissociates. NTN was induced with a rabbit polyclonal antibody. 3 h after disease induction, glomeruli were isolated and dissociated into single cells, and PLTs were identified by immunocytochemistry. One field from a representative glomerular cell dissociate from a nephritic rat is shown. PLTs were present singly or in aggregates (arrows)

both in close proximity to cells or non-cell associated. The large arrow shows a clump of PLTs. Inset, PLTs in close proximity to a PMN.

associated with PMNs were also noted (Fig. 4, inset). Defibrination moderately decreased the apparent numbers of PLTs: 18.0 ± 0.5 vs 11.0 ± 2.0 PLTs/100 cells in nephritic and defibrinated nephritic animals, respectively ($n = 3$, $P = 0.07$).

What was notable about these results taken together was the relative discrepancy between the decrease in total leukocyte and PLT influx with Fg depletion (~ 25 – 40%) and the more substantial decrease in proteinuria ($\sim 70\%$). This discrepancy

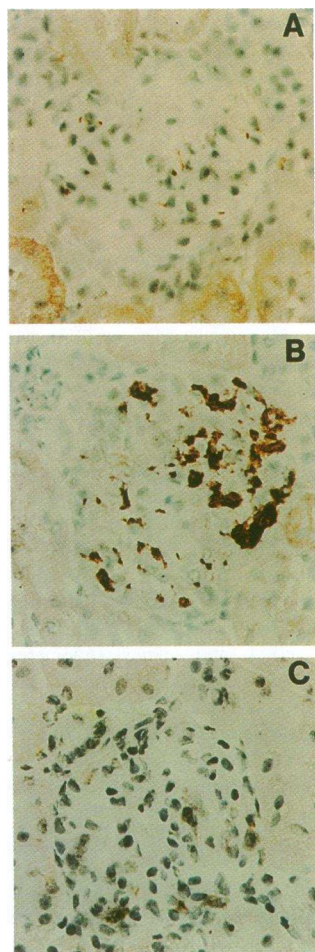


Figure 6. Glomerular expression of β_3 integrins during NTN: effect of PLT depletion. NTN was induced with a rabbit polyclonal antibody to rat GBM in unmanipulated and PLT-depleted animals. Nondisease controls were also assessed. Renal tissue was obtained 3 h after disease induction, and the expression of β_3 integrins was determined by immunohistochemistry. Representative glomeruli from one of three identical experiments are shown. A, normal control; B, nephritic animal; C, PLT-depleted nephritic animal.

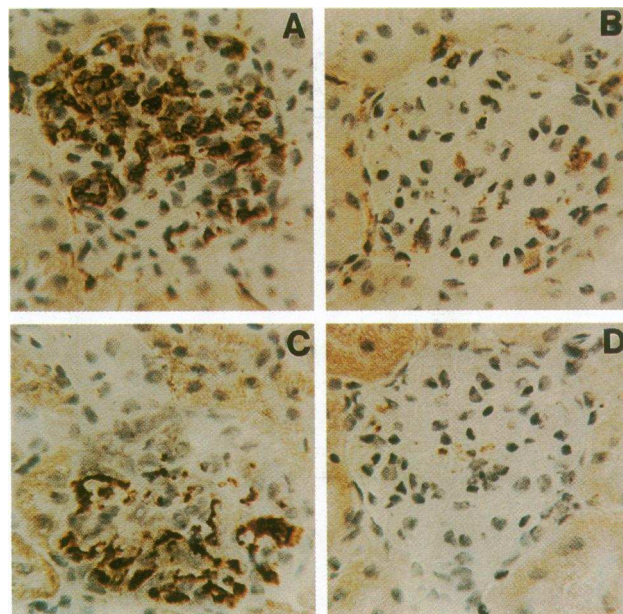


Figure 7. Glomerular expression of β_3 integrins during NTN: effect of PMN depletion. NTN was induced with a rabbit polyclonal antibody to rat GBM in unmanipulated and PMN-depleted animals. Nondisease controls were also assessed. Renal tissue was obtained 3 h after disease induction, and the expression of CD45 and β_3 integrins was determined by immunohistochemistry. Representative glomeruli from one of three identical experiments are shown. A and C, CD45 and β_3 integrin expression, respectively, in a nephritic animal; B and D, CD45 and β_3 integrin expression, respectively, in a PMN-depleted nephritic animal.

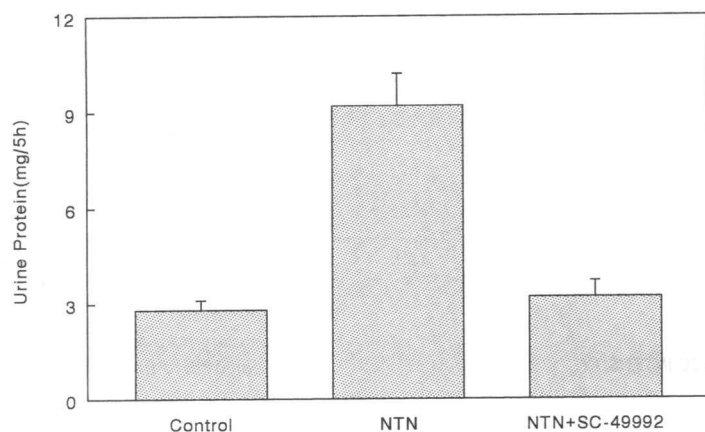
suggested that Fg might be more important to the in situ activation of PLTs and PMNs than their emigration from the vasculature into the glomerulus.

Effect of Fg receptor blockade on NTN. We were able to address this issue using the peptidomimetic of the RGD binding motif of Fg, SC-49992, which blocks Fg binding to PLT $\alpha_{IIb}\beta_3$ (17, 18). Because of the short half-life of this compound, we evaluated its effects when administered over the 3 h after disease induction. Similar to Fg depletion, PLT Fg receptor blockade largely attenuated the proteinuria which occurred in NTN (by $\sim 90\%$, Fig. 5). However, in contrast to Fg depletion, SC-49992 had little effect on either total leukocyte or PMN influx into glomeruli at 3 h ($< 10\%$ decrease, Fig. 5). We did observe a tendency towards a decline in PLT counts within glomerular cell dissociates with SC-49992: 20.0 ± 2.0 vs 13.0 ± 2.0 PLTs/100 cells in nephritic and SC-49992-treated nephritic animals, respectively, though this was not statistically significant ($n = 3$, $P = 0.18$).

These data reinforced the impression that Fg was critical to the in situ activation of PLTs and PMNs within the glomerulus. Moreover, they suggested that expression of $\alpha_{IIb}\beta_3$ was an essential component of this coactivation response.

β_3 integrin expression in NTN. To understand this issue, we next addressed the expression of β_3 integrins within glomeruli during the acute phase of NTN. In normal animals, there was no evident expression of β_3 integrins by glomerular cells, although there were occasional small positively staining cellular fragments (presumably PLTs) within the vasculature (Fig. 6 A). By 3 h after disease induction, however, expression of β_3 integrins was dramatically increased in the glomerulus (Fig. 6 B). Our initial presumption was that this increased expression was

A



B

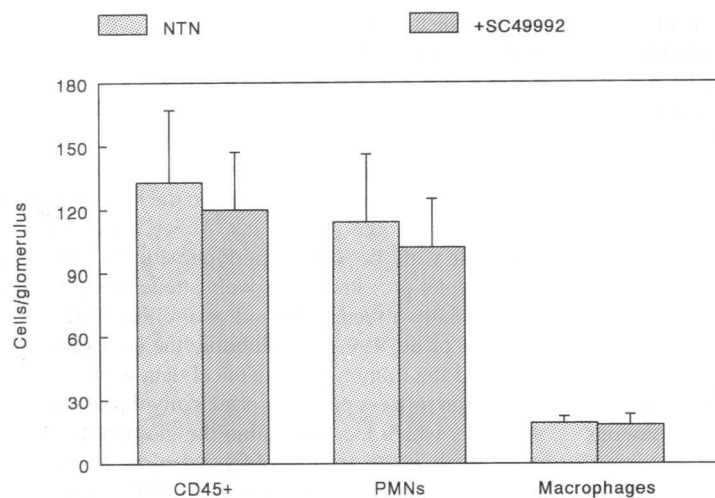


Figure 5. Effect of SC-49992 on NTN. NTN was induced with a rabbit polyclonal antibody to rat GBM in animals infused with either SC-49992 or saline (i.e., vehicle) intravenously. Nondisease controls were also assessed. Urine was collected for a period of 3 h for determination of proteinuria. Glomeruli were then isolated from nephritic animals (with or without SC-49992) and dissociated into single cells, and leukocytes were quantified and subtyped by immunocytochemical staining. **A**, proteinuria in normal controls, nephritic animals, and nephritic animals treated with SC-49992 ($n = 4, 7,$ and 6 , respectively). Proteinuria was significantly different in nephritic animals relative to the other two groups ($P < 0.05$). **B**, leukocyte content (total, PMNs, and macrophages) of glomeruli from nephritic animals and nephritic animals treated with SC-49992. $n = 4$ for both.

a function of PLT deposition, and, in consequence, we subsequently examined the effect of PLT depletion on the increase in β_3 integrin with NTN. As shown in Fig. 6 C, PLT depletion completely attenuated the increase in β_3 integrin expression.

However, in light of our previous results which show a cooperative interaction between PMNs and PLTs with respect to migration (6), we additionally addressed the effect of PMN depletion on β_3 integrin expression within glomeruli. As shown in Fig. 7, A and B, PMN depletion markedly attenuated the influx of PMNs into the glomerulus as exhibited by staining for CD45. Moreover, PMN depletion, like PLT depletion, also completely prevented the increase in β_3 integrin expression in acute NTN (Fig. 7, C and D).

These immunohistologic findings demonstrated that the increased glomerular β_3 integrin expression was a function of both PLTs and PMNs but did not definitively address alterations in expression at a cellular level. We broached this issue by

performing FACS® on glomerular cell dissociates using our anti- β_3 integrin monoclonal. As shown in Fig. 8 A, numerous CD45-positive cells were present in inflamed glomeruli. However, staining of the identical preparation with the anti- β_3 integrin monoclonal showed a small population of positively staining cells of varying intensity. Using immunocytochemistry we were able to identify the relatively more intensely stained particles as PLTs which were both free and adherent to other cells similar to that shown in Fig. 4 (data not shown). β_3 integrin expression by PMNs or mesangial cells was modest by immunocytochemistry and no alteration in staining was evident with nephritis with this method (data not shown).

Although defibrination markedly diminished proteinuria, β_3 integrin expression was not noticeably affected by this manipulation (data not shown). This result is consistent with our aforementioned observations that defibrination only modestly altered PLT influx.

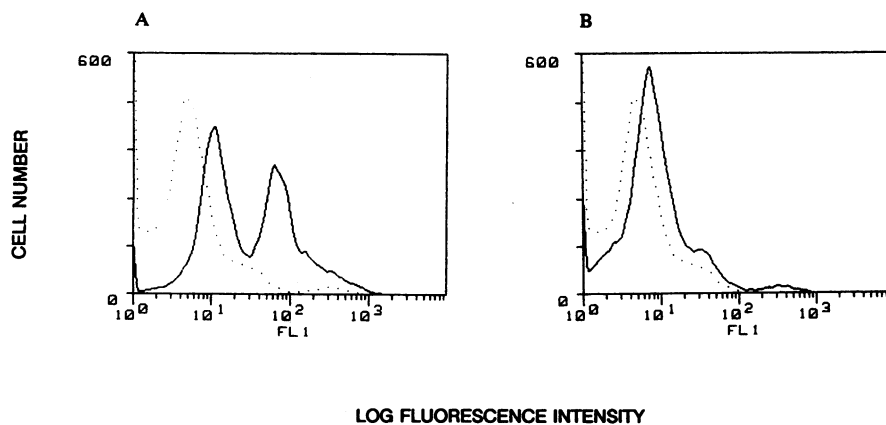


Figure 8. CD45 and β_3 integrin expression in dissociated glomerular cells from inflamed glomeruli. Glomeruli from nephritic animals (3 h after disease induction) were dissociated into single cells, and CD45 (A) and β_3 integrin expression (B) were determined by FACS®. Solid lines represent the inclusion of primary antibody; dotted lines represent its omission (i.e., nonspecific binding of the secondary antibody). This experiment was repeated three times with similar results.

Fg and PLT-PMN activation in vitro. These *in vivo* studies suggested that Fg contributed to glomerular damage in NTN (as manifested by proteinuria) via the upregulated expression of $\alpha_{\text{IIb}}\beta_3$ which resulted from PLT-PMN influx into glomeruli. To understand in more specific terms how Fg might contribute to proteinuria, we examined PLT-PMN activation *in vitro* and the effect of Fg receptor blockade on this process. We focused on the PMN respiratory burst given prior studies implicating this process as a proximate cause of proteinuria in immune-mediated glomerular injury (22), and given data suggesting that Fg can mediate PLT-PMN coactivation *in vitro* (23, 24).

PMNs incubated alone or with thrombin were quiescent in terms of the respiratory burst (Fig. 9 A). When PLTs and PMNs were cocultured, a modest increase in the respiratory burst was noted (possibly in part resulting from PLT activation during isolation, Fig. 9 A). However, when thrombin was added to PLT-PMN cocultures, a threefold increase in superoxide formation was seen (Fig. 9 A). This stimulatory effect of thrombin in PLT-PMN cocultures was inhibited by ~50% by Fg receptor blockade (Fig. 9 A). Of interest, although Fg receptor blockade consistently inhibited PLT-PMN coactivation in terms of the respiratory burst, we noted that SC-49992 (but not anti- β_3 antibody) inhibited the visually apparent heterologous aggregation.

PMA, in contrast to thrombin, directly stimulated the PMN respiratory burst (Fig. 9 B). No further enhancement of this response was seen when platelets were added to the incubation (data not shown). SC-49992 modestly inhibited this response (~25%), whereas anti- β_3 antibody had little effect (Fig. 9 B).

Discussion

PMN-PLT interactions appear to play a critical role in the development of the acute inflammatory response and the resultant tissue damage and dysfunction (8, 9). In our previous study and this current work, we have specifically corroborated this hypothesis within the context of immune complex-mediated glomerulonephritis. As we have observed, PLTs and PMNs colocalize to the glomerulus in the acute phase of NTN and become coordinately activated, resulting in glomerular dysfunction and a marked change in glomerular eicosanoid production (6). The intent of the current work was to begin to delineate the molecular mechanisms which potentially mediate both the localization of these cells to the glomerulus and their activation *in situ*.

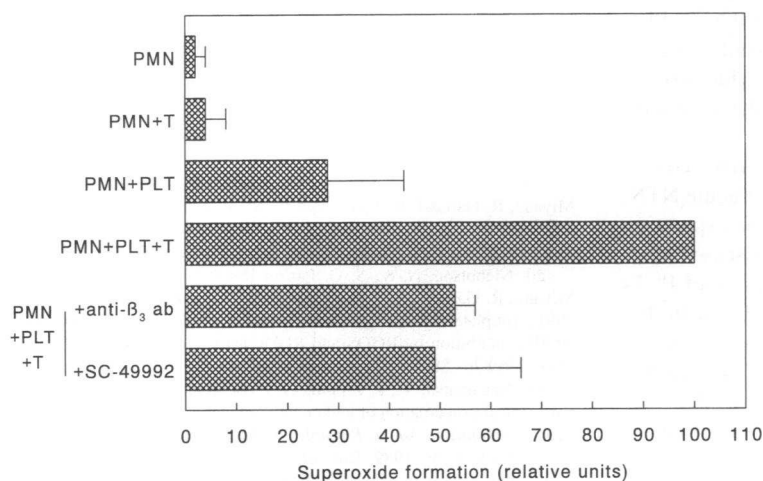
This study would suggest that Fg plays an important role in both of these processes in that defibrination substantially attenuated the acute glomerular damage in NTN (as manifested by proteinuria) and significantly, though less substantially, decreased the accompanying influx of PMNs and PLTs into glomeruli. These results corroborate and expand on earlier work which showed that defibrination or induction of fibrinolysis could ameliorate immune complex-mediated glomerulonephritis both histologically and functionally although this effect was not invariable (10, 11). The variability seen in these earlier studies may have related in part to the persistence of Fg within PLTs (16), an issue which we circumvented by pretreating animals for 5 d before disease induction.

It would appear from the current data that the major role of Fg in acute NTN is to participate in the PLT-PMN coactivation process within the glomerulus. As we found, PLT Fg receptor blockade with the RGD peptidomimetic, SC-49992, in contrast to Fg depletion, rather selectively attenuated proteinuria without substantially affecting the influx of either PMNs or PLTs. The efficacy of SC-49992 in inhibiting the development of proteinuria and the immunocytochemical data would suggest that this activation process proceeds principally through $\alpha_{\text{IIb}}\beta_3$ expressed by PLTs which localize within the glomerulus. We would further conclude that the role of Fg and $\alpha_{\text{IIb}}\beta_3$ in glomerulonephritis is to mediate PLT activation of the PMN respiratory burst *in situ* and thereby proteinuria. As we found in our *in vitro* studies, Fg receptor blockade attenuated PLT-dependent PMN production of reactive oxygen species, reactive oxygen species being one of the proximate causes of proteinuria in acute immune-mediated glomerulonephritis (22).

Our conclusions regarding the role of Fg and $\alpha_{\text{IIb}}\beta_3$ in NTN, however, are subject to the caveat that SC-49992 is not absolutely specific for the PLT Fg receptor $\alpha_{\text{IIb}}\beta_3$. SC-49992 can block other receptors recognizing the RGD motif such as the vitronectin receptor ($\alpha_v\beta_3$) or the leukocyte response integrin (another member of the β_3 integrin family), albeit less well (references 17, 18, and 25-27, and Gresham, H., personal communication). We, in fact, noted that even the direct activation of the PMN respiratory burst with PMA could be partially inhibited with SC-49992, implying that the aforementioned β_3 integrins on PMNs may also participate in the proinflammatory effects of Fg in glomerulonephritis.

PLT-PMN interactions can be bi-directional, though PLT-mediated PMN activation and PMN-mediated PLT activation both appear to require surface contact and to be mediated by

A



B

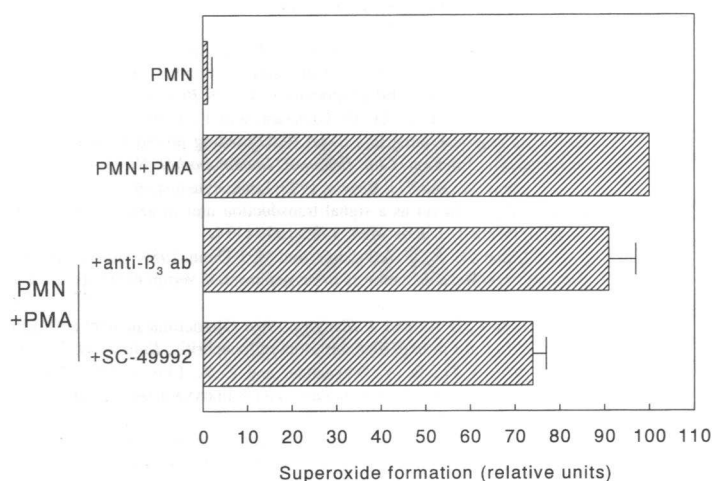


Figure 9. PLT-PMN activation in vitro: role of Fg and β_3 integrins. PMNs were incubated alone or in the presence of PLTs. Thrombin (T) or PMA was added as an agonist. To block the Fg receptor, cells were preincubated with SC-49992 or anti- β_3 antibody before stimulation. The PMN respiratory burst was measured as superoxide formation quantified using cytochrome *c*. Results are presented as arbitrary units relative to agonist stimulation (=100). **A**, thrombin-stimulated PMN-PLT incubations. All groups were significantly less than thrombin-stimulated PLTs+PMNs ($P < 0.05$), except for PLTs+PMNs+thrombin+SC-49992 for which $P = 0.09$. $n = 3$ for all groups. **B**, PMA-stimulated PMN incubations. SC-49992 treatment significantly inhibited PMA-stimulated PMN superoxide production ($P < 0.05$, $n = 4$ for all groups). PMA-stimulated PMN superoxide formation was approximately twofold greater than thrombin-stimulated PMN-PLT incubation superoxide formation.

Fg (23, 24). In light of the marked increase in glomerular lipid mediator production in the context of NTN (28–30), it is further noteworthy that lipid autacoids may be involved in this reciprocal interaction. Thromboxane may stimulate PLTs to activate PMNs, leukotriene B_4 may stimulate PMNs to activate PLTs, and PMN-derived platelet activating factor may serve to activate PLTs (23, 24). However, with respect to NTN, prior studies would suggest that platelet activating factor, thromboxane, and leukotriene B_4 independently play modest roles in the coactivation process (21, 31, 32).

Although Fg appears to be critical to PLT-PMN coactivation, the present data also suggest that Fg may play a role in PMN-PLT comigration into glomeruli, albeit a relatively more limited one. Hypothetically, Fg may act as a ligand for PMN β_2 integrins or intercellular adhesion molecule and facilitate their transendothelial migration and/or Fg may serve as a molecular bridge between PLTs and PMNs (13). It is worth noting that Fg binding to CD11b/CD18 is not mediated by the RGD motif (13) and thus would not be blocked by SC-49992.

However, it is clear that Fg-independent mechanisms are important for the influx of PMNs and PLTs into glomeruli in

NTN. Of relevance to this comment, we observed that PLT-PMN aggregation in vitro was not invariably inhibited by Fg receptor blockade even though the respiratory burst was. Based on the available data, we would surmise that P-selectin/GMP-140 might be important to this process (33, 34), though we have not been able to substantiate this conclusion using the anti-P selectin monoclonal antibody, PB1.3 (kindly provided by J. Paulson, Cytel, San Diego, CA) (references 35 and 36, and Wu, X., and J. B. Lefkowitz, unpublished data). However, it is possible that other molecules, such as complement components (i.e., C3b and C3bi), might serve to promote PLT-PMN adhesion, bypassing P-selectin (8, 9). Understanding the mechanisms by which PLTs adhere to PMNs as they emigrate from the vasculature into the mesangium will require further investigation.

Although the mechanisms of concern for the current study deal with events which occur early in the inflammatory cascade, acute events clearly have important ramifications for chronic outcomes. Within the context of NTN, studies have shown that the magnitude of the acute insult to the glomerulus, if sufficiently severe, can result in glomerulosclerosis (37). Moreover,

PLTs contain a panoply of mediators which may contribute to perpetuation of the inflammatory response and contribute to the process of glomerulosclerosis such as PDGF, FGF, TNF α , PF4, and TGF β (37–40). Thus, developing an understanding of the molecular mechanisms operative early on may provide insights into strategies which may be useful to prevent the long-term adverse outcome of glomerular scarring.

In conclusion, Fg appears to play a critical role in the coactivation of PLTs and PMNs within the glomerulus in acute NTN. This activation is most likely mediated via $\alpha_{IIb}\beta_3$, the expression of which is markedly increased in glomeruli after disease induction. This altered expression appears due to the influx of PLTs in a PMN-dependent fashion which in part is mediated by Fg, but which clearly is dependent to a large degree on other molecular interactions which remain to be determined. Developing an understanding of these acute events may help in the development of strategies not only to ameliorate acute glomerular injury, but also to prevent glomerular scarring which may result from the initial insult.

Acknowledgments

This work was supported by National Institutes of Health grant DK-37879. Dr. Lefkowitz is a Burroughs-Wellcome Scholar in Clinical Pharmacology. Dr. Horton is supported by the Imperial Cancer Research Fund (U.K.).

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