Neutropenia impairs venous thrombosis resolution in the rat

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Objectives: **Neutrophil influx is one of the first events in a formed deep venous thrombosis (DVT), but whether these cells are active participants in the resolution process is not clear. This study tests the hypothesis that neutrophils (PMN) are active participants in DVT resolution.**

Methods: **Thrombosis was induced by inferior vena caval (IVC) ligation in male Sprague-Dawley rats, and rats were sacrificed at 2, 4, or 7 days for evaluation of the thrombus. Neutropenia was induced by rabbit anti-rat PMN serum, and controls received rabbit serum. Venography was performed at the 7-day time point. Immunohistochemical staining was performed to quantify intrathrombus PMNs and monocytes, and the myeloperoxidase (MPO) assay was performed to assess intrathrombus neutrophil activity. Intrathrombus concentrations of kerotinocyte cytokine (KC), macrophage inflammatory protein-2 (MIP-2), γ interferon inducible protein-10 (IP-10), macrophage inflammatory protein-1 α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor (TNF)–α were quantified by enzyme immunoassay at each time point and normalized to total protein. Total collagen was determined at day 7.**

Results: **Peripheral blood smears showed a 94% PMN reduction at 2 days ($P < .05$), recovering to 44% of control at 7 days. Intrathrombus PMNs were significantly lower in neutropenic rats at 2 and 4 days, but there were no differences in intrathrombus monocytes. The MPO assay confirmed reduced neutrophil activity at 4 days. Thrombi from neutropenic rats were larger at 2 and 7 days compared with controls. In vivo thrombus area at 7 days as assessed by venography was also greater in neutropenic rats as compared with controls. The intrathrombus KC concentration was increased more than 20-fold in the neutropenic rats at 2 days, but there were no significant differences in other intrathrombus chemokines. Finally, intrathrombus collagen was increased over threefold in neutropenic rats as compared with controls.**

Conclusion: **Neutropenia impairs DVT resolution by several measures, most likely by altering normal fibrinolytic activity and thrombus collagen turnover. (J Vasc Surg 2003;38:1090-8.)**

Deep venous thrombosis (DVT) is a common condition affecting approximately 250,000 patients per year and responsible for 50,000 deaths per year.[1] Although anticoagulant therapies are effective for prophylaxis and in preventing thrombus propagation, the basic mechanisms of DVT resolution have only recently become better understood. Systemic neutropenia and deficits in neutrophil activity are common effects of many diseases, such as chemotherapy-induced leukopenia and viral infections. These patients are at higher risk of DVT, although the complete mechanisms have not been delineated. A recent retrospective study has suggested that patients with malignancy and recurrent leukopenic episodes are at increased risk of recurrent venous thromboembolic events.[2]

These clinical observations indirectly argue for the importance of neutrophils (PMN) in clearance of thrombus. Indeed, PMNs are associated with thrombus resolution, as first demonstrated by Stewart,[3] and explored in previous studies from our group.[4] A primary function of PMNs is in modulating an inflammatory response by means of cytokines, proteases, and reactive oxygen species. Although PMNs may be prothrombotic at DVT initiation, their role may be important for thrombolysis once it has formed, through direct and indirect cellular mediators.[5,6]

Localization and activation of PMNs and other portions of the cellular immune system are mediated by chemokines, which are chemotactic cytokines with varying specificities and roles.[7] Chemokines are classified on the basis of the placement of four cysteine residues toward the beginning of their protein sequence, and the best studied are the CC and CXC subfamilies. The CXC chemokines are divided into ELR+ and ELR− subfamilies, on the basis of the presence of a three amino acid sequence preceding the CXC sequence. Of note, CXC ELR+ chemokines are generally neutrophil-attractant and proangiogenic; interleukin 8 (IL-8) is the prototypical member of this group. Moreover, CXC chemokines may play a direct role in DVT resolution in a murine model (unpublished data).

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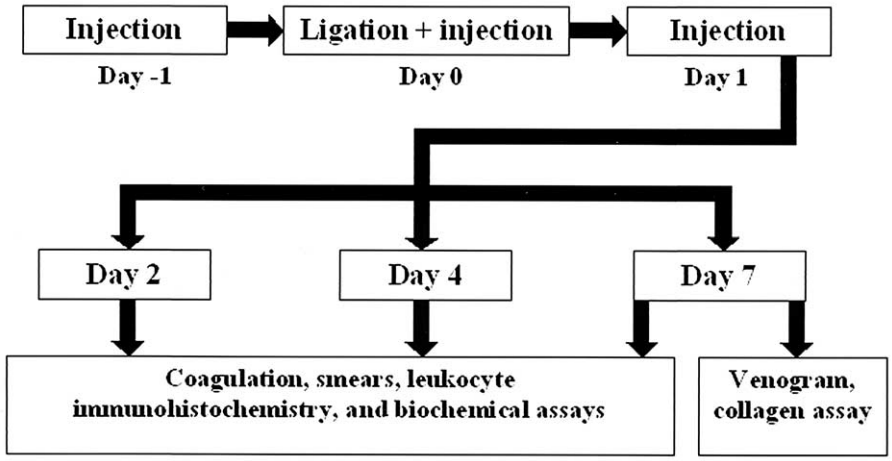

Fig 1. Study design. This chart details the sequence of rabbit anti-rat PMN serum (anti-PMN serum) or control rabbit serum injection, IVC ligation, sacrifice, and the outcome measurements assessed in this study.

Given the potential roles of inflammatory cells and mediators in initiating and propagating venous thrombosis, several anti-inflammatory approaches to limiting DVT formation, such as P-selectin antagonism,[8-10] anti–tumor necrosis factor (TNF) antibody,[8] and supplementation of interleukin 10 (IL-10) by rIL-10 injection or local overexpression[11,12] have been studied. These agents generally have been administered before thrombus formation to produce an anti-inflammatory milieu, with thrombus formation subsequently attenuated. However, proinflammatory states are beneficial in many settings of established injury; indeed, inflammatory responses are an essential part of wound healing.[13,14] In a previous report, IL-8 supplementation accelerated DVT resolution in the rat model, but whether this was conferred by the chemokine itself or the increased intrathrombus PMNs was not established.[15] This study sought to determine whether PMNs play a direct role in DVT resolution and whether PMN depletion at the time of DVT genesis secondarily alters the local leukocyte and chemokine environment.

METHODS

Study design and animal model. The study design is summarized in Fig 1. Male Sprague-Dawley rats (350 to 450 g) were used for all studies. Thrombosis was induced in a rat model that has been used successfully by our laboratory, and it is fully described elsewhere.[8,9,11,12,15] Briefly, the model consists of ligation of the inferior vena cava (IVC) below the renal veins and of all visible side branches. A clot develops in >90% of rats. At sacrifice, a blood sample was taken from the suprarenal IVC or left ventricle, and the thrombosed IVC was carefully dissected and removed for histologic and biochemical studies after thrombus-vein wall separation, as follows. Thrombus weight was measured and corrected for length of segment as described.[8-12]

Both neutropenic and control groups were studied at each time point, and rats were sacrificed at 2, 4, or 7 days thrombus induction. To induce systemic neutropenia, rats allocated to neutropenia groups were given a 0.25-mL to 0.5-mL loading dose of rabbit anti-rat PMN serum (Accurate Chemical, Westbury, Conn) by means of tail vein injection the day before IVC ligation and 0.25-mL boosters on the day of ligation and the day after ligation.[16] Rats allocated to control groups were given the same volumes of rabbit serum at the same time points. Systemic neutropenia was confirmed by analyzing a peripheral smear stained by Diff-Quik (Jorgensen Laboratory, Loveland, Colo) at sacrifice, and random animals had venous blood sampled for thrombin clotting time (TCT) and activated partial thromboplastin times (aPTT) per standard techniques.

All studies were approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA #7916) and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Venography. Venography was performed by using a BV29 C-arm fluoroscopy unit (Phillips Medical System, Cincinnati, Ohio) with the image intensifier placed ~40 cm above the table to provide adequate magnification. The rats were positioned in the center of the field, and 1.5-mL Hypaque solution (2.5-mL Hypaque with 1.5-mL saline) per radiograph was injected into the tail vein. The subtraction mode was used to shoot the final images. The contrast was visualized under real time to assure adequate filling of the IVC. Anterior-posterior and lateral radiographs were taken by rotating the animal and not the C-arm. The digital venogram was then transferred onto a computer hard drive by using the software Vidcap (Version 5.1; Seattle, Wash) and then analyzed by using NIH Image 1.62, a public domain program. The images had resolution of (250 pixels/cm) and were converted to TIFF format with Adobe Photoshop (Adobe Systems, Inc, Seattle, Wash). Note that the veterbrae of the animals were used to standardize the thrombus measurements to allow intra-animal comparison and that the measurements were quantified as millimeters

squared. Review of the images was done in a blinded fashion.

Immunohistochemistry. The harvested proximal one third of the thrombus, closest to the ligature, was consistently used for histologic analysis. The IVC was imbedded in paraffin, sliced into 10-μm-thick sections, and staining was performed as described.[12,15] Briefly, the tissue was deparaffinized and dehydrated through graded alcohols, followed by blocking nonspecific binding sites with species-specific serum. Neutrophils were localized with 1:1000 anti-PMN antibody (Accurate Chemical, Westbury, Conn), and monocytes were localized with 1:100 ED-1 antibody (Serotec, Oxford, England). A rabbit kit was used for the neutrophil staining, and a mouse kit was used for the monocyte staining per manufacturer's instructions (both from Vector Laboratories, Burlingame, Calif). Slides were counterstained with hematoxylin and cover slipped. For analysis, five randomly chosen high-powered fields were selected from near the vein wall–thrombus interface (as most leukocytes are present in this area), and positive staining cells were counted in a blinded fashion.

Myeloperoxidase (MPO) assay. Thrombus samples were prepared with separation of the thrombus from the vein wall, followed by homogenization, sonication (30 s), and centrifugation (15 min at 10000 × *g*) of the thrombus in a buffer solution. The homogenate was then reacted with *o*-dianisidine hydrochloride (Sigma, St. Louis, Mo), and the reaction product was measured at 490 nm as described.[11] Results were reported as change in optical density per minute.

Enzyme-linked immunosorbent assays (ELISA). ELISAs were performed as previously described for kerotinocyte cytokine (KC, a rodent IL-8 analogue), macrophage inflammatory protein-2 (MIP-2), γ interferon inducible protein-10 (IP-10), macrophage inflammatory protein-1α (MIP-1α), monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor–α (TNF-α).[12,15,17] The thrombi were prepared in the same manner as for the myeloperoxidase assay. Tissue homogenate ELISAs were done with species-specific primary antibodies quantified by using a double ligand technique. Plates were read at 450 nm.

Total protein quantification was performed by a modified Bradford assay (Pierce, Inc, Rockford, Ill) per manufacturer's instructions on the homogenate of each animal sample used for ELISA, with serial dilutions of bovine serum albumin as standards. Results were expressed as pg/mg protein, to account for differing protein compositions of samples.

Collagen assay (Sircol collagen assay). Thrombus collagen content was estimated by a commercially available kit according to manufacturer's instructions (Bicolor LTD, Belfast, North Ireland). This collagen assay uses a quantitative method by which anionic sirius red (direct red) dye binds to the side chains of basic amino acids found in collagen. Total collagen is presented as μg/mg thrombus.

Statistical analysis. All statistical analysis in this study was performed by one-way analysis of variance or *t* test, and data are reported as mean ± SEM. $P < .05$ was considered significant.

RESULTS

Rats treated with anti-PMN serum were neutropenic during thrombus formation and through sacrifice. To confirm the effects of the anti-PMN serum, peripheral blood smears were performed. A 94% reduction in circulating PMNs was seen among neutropenic rats sacrificed at 2 days compared with controls from the same time point (control 21 ± 2 vs 1.4 ± 0.0% differential, $P < .05$, N = 3), and a 44% reduction among neutropenic rats compared with time-matched controls was seen at 7 days (control 32 ± 7 vs 14 ± 2% differential, $P > .05$, N = 3). No significant differences in peripheral monocyte counts were observed. Lastly, thrombin clotting time and activated partial thromboplastin time, as a measure of coagulability, were not significantly different between control and anti-PMN rats (control aPTT/TCT = 36 ± 4/28 ± 4 vs anti-PMN 32 ± 5/22 ± 2 seconds, $P > .05$, N = 6).

Immunohistochemical staining was used to localize PMNs and monocytes in the thrombus. The number of PMNs detectable in the thrombus was over 51% lower in rats treated with anti-PMN serum rats at 2 days (18.2 ± 6 vs 37.4 ± 6.5 cells/5 hpf, N = 5; $P < .01$) and over 41% lower at 4 days (23 ± 9 vs 39 ± 4 cells/5 hpf, N = 5; $P < .01$) compared with controls at those time periods. The difference between anti-PMN and control PMN counts (15.4 ± 4.2 vs 18.4 ± 6.2, N = 4) rats at 7 days was not significant, with a noted decrement in intrathrombus PMN content but none in the anti-PMN rat thrombi (Figs 2, *A* and 3). There was no difference in the number of intrathrombus monocytes detectable at 2, 4, or 7 days among rats treated with anti-PMN serum (N= 4) (Fig 2, *B*). The MPO activity reflects PMN function as measured by enzyme activity, and it indirectly measures PMN content. No significant difference was observed at the 2-day time point (N = 3), whereas a significant reduction in activity was observed at the 4-day time point (13 ± 7 vs 36 ± 2 U activity, n = 3; $P < .05$) (Fig 2, *C*).

Thrombus size was larger in neutropenic rats. Thrombus size was measured at sacrifice as a gross measure of thrombus resolution. It is assessed by calculating the weight/length ratio, which reflects the density of the thrombus, accounts for variations in the length of vein recovered at sacrifice, and has been consistent in reflecting thrombus dissolution.[8,9,11,12,15] Weight/length ratios of thrombi from neutropenic animals harvested at 2 days (147 ± 12 vs 94 ± 10 mg/cm, n = 6; $P < .05$) and 7 days (168 ± 71 vs 121 ± 18 mg/cm, n = 6; $P < .05$) were significantly larger than control animals' thrombi (Fig 4). This effect was lost among animals sacrificed at 4 days, with control rats having a weight/length ratio of 128 ± 14 mg/cm and neutropenic rats having a weight/length ratio of 106 ± 9 mg/cm (N = 6, $P = .20$).

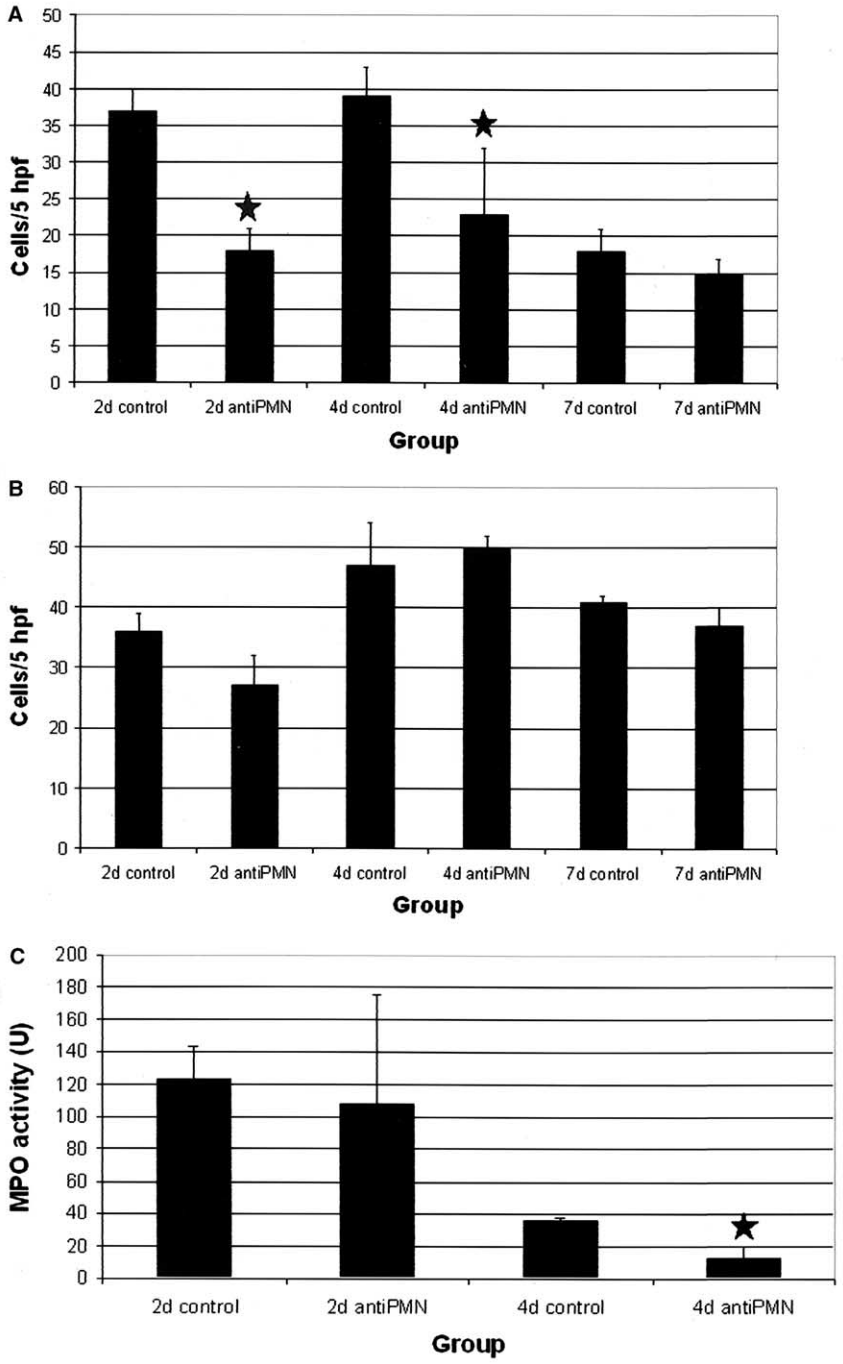

Fig 2. The inflammatory response within the thrombus was assessed by immunohistochemical staining for (**A**) PMNs and (**B**) monocytes, as well as the (**C**) MPO assay. (**A**) There were significantly fewer intrathrombus PMNs in neutropenic rats at 2 and 4 days (both $P < .05$), but no difference at 7 days. (**B**) There were no differences in intrathrombus monocytes among any groups. (**C**) There was significantly less MPO activity among 4-day neutropenic rats compared with 4-day controls, but no differences were detectable at other time points.

Thrombus area in vivo is increased in neutropenic rats. To corroborate the findings of increased thrombus size at harvest, venography was performed to allow assessment of intrathrombus area in two dimensions at 7 days. Representative venograms are shown in Fig 5, which reflects the increased in vivo thrombus area in neutropenic rats. The mean area was 10.4 ± 1.3 mm^2 in the control rats and 19.1 ± 2.6 mm^2 among the neutropenic

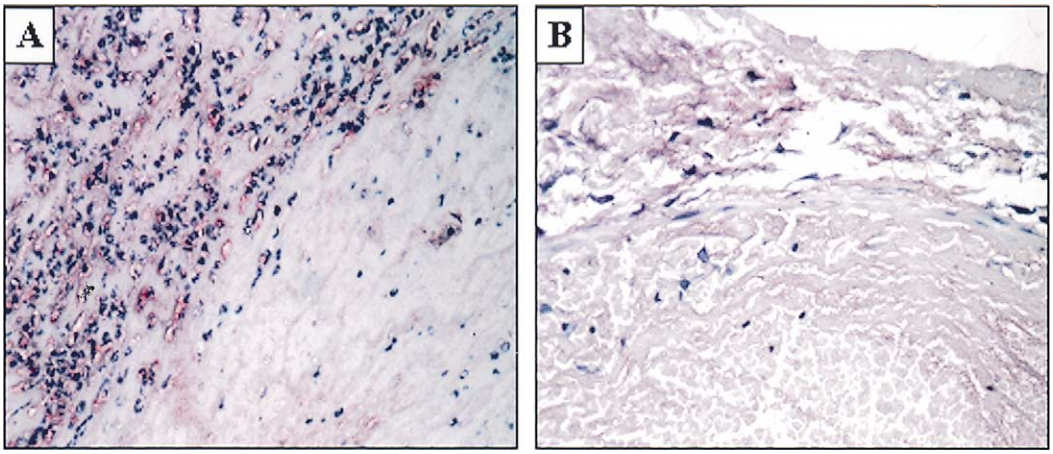

Fig 3. Representative immunohistochemical staining of thrombi from (**A**) control and (**B**) neutropenic rats (200×). There are far fewer PMNs visible within the thrombi of neutropenic rats.

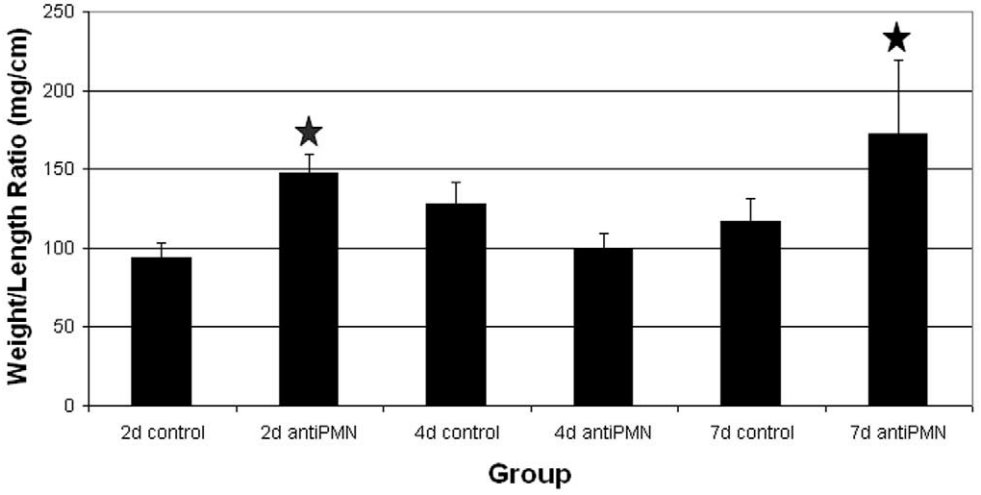

Fig 4. Thrombus size. Neutropenic rats had thrombi with larger weight/length ratios at 2 days and 7 days ($P < .05$), but there was no difference in thrombus size at 4 days.

rats ($P < .05$, N = 5-7), which reflects an increased area of occlusion within the same region of interest analyzed at 7 days.

Intrathrombus KC concentration is greater in neutropenic rats. As chemokines are involved with directing leukocytes to areas of inflammation as well as inducing intrinsic leukocyte activity, common CXC and CC chemokines in control and neutropenic rats were analyzed. Chemokines may be produced by infiltrating leukocytes as well as resident vein wall cells. Intrathrombus KC concentration was nearly 20-fold higher at 2 days in neutropenic rats as compared with controls (200 ± 87 vs 8.9 ± 8 ng/mg protein, N = 5, $P < .05$). The cytokines TNF-α, MIP-2, and IP-10 were all present in very low amounts (<50-pg/mg protein) in both groups (N = 4 each). Two CC chemokines, MIP-1α and MCP-1 showed no significant differences at 2 or 7 days between the treatment groups (N = 4, $P > .10$). However, 4-day thrombus MIP-1α was 2.3-fold higher in controls as compared with anti-PMN rats (277 ± 61 vs 118 ± 56 pg/mg protein, N = 8, $P = .08$). More significantly, 4-day MCP-1 was twofold higher in control thrombi than anti-PMN treated rats (89 ± 21 vs 34 ± 7 pg/mg protein, N = 8, $P = .02$).

Neutropenic rat thrombi have increased fibrosis. As the thrombus matures, acute phase collagen turnover is apparent.[18] Total collagen within the thrombus was measured to assess the extent of fibrosis remaining within the thrombus at 7 days. There was 3.3-fold greater collagen in the neutropenic rat thrombi as compared with controls ($P < .05$; N = 5, Fig 6, *A*), which suggests impaired collagen turnover in the neutropenic rats. Trichrome staining of the thrombus sections shows intrathrombus collagen (Fig 6, *B,C*).

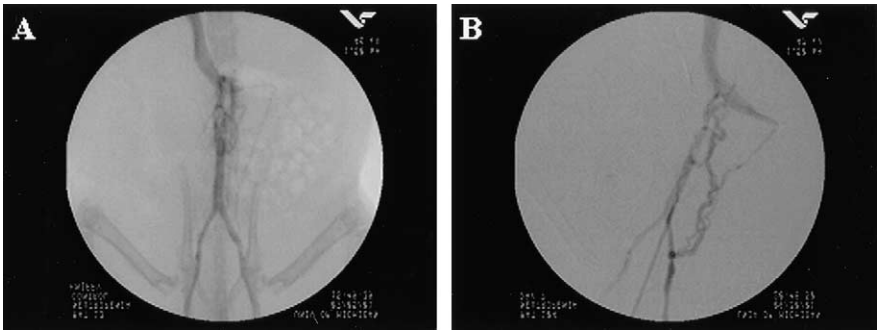

Fig 5. Venograms from (**A**) control and (**B**) neutropenic rats taken at 7-days post-IVC ligation. (**A**) The thrombus of the control rats had greater recanalization, and less IVC thrombus area, which was not seen in the neutropenic rats (**B**), where larger IVC thrombus area and more extensive collateral formation was seen.

DISCUSSION

This study confirms the important role of PMNs on DVT resolution, which previously in vivo has only been associative,[3] as neutropenic rats had larger thrombus size and in vivo area as well as increased fibrosis. The impaired thrombus resolution in neutropenic rats was likely solely caused by the PMN, as peripheral and thrombus monocyte (ED-1) counts were similar between groups. No differences in baseline clotting times were observed, which argues against a systemic effect of the anti-PMN serum. Moreover, it was unlikely caused by alterations in common chemokines, as only KC (a primary CXC chemokine with PMN chemoattractant activity) was increased in the neutropenic rats. This finding also indirectly suggests that KC, which has in vitro proangiogenic activity, did not accelerate thrombus resolution outside of PMN recruitment and perhaps activation. This finding also broadens prior work, whereby IL-8 supplementation significantly enhanced DVT resolution in the same rat model and was associated with increased intrathrombus PMNs.[15] Moreover, IL-8 supplementation in the setting of PMN depletion in the rat model of DVT did not reverse the impaired resolution (unpublished observations). Taken together, these observations suggest the PMN is an early primary cellular effector of DVT dissolution. However, it is also possible because PMN was depleted at the time of thrombus generation that the thrombus composition might be entirely different. However, the basic gross histology was not markedly different and monocytes (ED-1+ cells) were not significantly fewer.

The finding that intrathrombus PMNs were significantly decreased as compared with controls but 2-day MPO was not as greatly reduced suggests that those PMNs within the thrombus of the neutropenic rats may have been maximally activated by the increased chemokine KC. However, although the 2-day neutropenic rat's intrathrombus PMNs may have been activated, it does not necessarily follow that this activity is reflected as DVT resolution. Chemokines are known to have PMN actions beyond simply chemoattractant activity.[7,14,17] A paradoxical increase in KC with neutrophil depletion has previously been shown for KC mRNA expression in abdominal wounds[14] and in KC protein levels in a CXCR2 deletion mouse model of cerebral abscess and DVT[19] (unpublished data). Neutrophil depletion seemed only to alter the CC chemokines MCP-1 and MIP-1α at day 4, although thrombus monocyte influx was not impaired. It is speculated that as the thrombi had fewer PMNs, it may be that certain mediator interactions between the thrombus and vein wall were altered and suggests the interplay of surrounding vein wall and other leukocyte subtypes. How this decrease in these two CC chemokines may have impaired monocyte function is unknown.

One potential mechanism to explain why PMN depletion impairs DVT resolution includes loss of natural early fibrinolytic activity, and the larger thrombi in the PMN-depleted rats supports this, although it may be biphasic, as the 4-day thrombi were not significantly different in size. Alternatively, the early influx of PMNs may be important for fostering thrombus matrix changes that mediate activity of other leukocytes. For example, the PMNs may favorably alter the thrombus matrix for monocyte release of angiogenic mediators.[20] Neutrophils release a plasminogen activator (PA), separate from known PAs such as tissue plasminogen activator.[6] Recent work for Moir and colleagues has shown in an in vitro system that PMNs mediate endogenous spontaneous fibrinolysis probably via urokinase PA.[21] PA inhibitor-1 (PAI-1) is the principal inhibitor for several PAs, and PAI-1 is degraded by neutrophil elastase.[22] Additionally, the plasmin inhibitor, α2-antiplasmin, is oxidized and inactivated by PMNs.[23] Taken together, it is likely that the PMN contributes to early DVT resolution by direct and indirect promotion of a fibrinolytic environment. Lastly, there is no data in the literature to support the notion that PMNs that release more MPO (eg, in the 2-day anti-PMN treated rats) are more active with regard to fibrinolysis in contrast to the known indirect fibrinolytic activity of elastase.[22]

A second possible mechanism that could explain impaired DVT resolution in neutropenic rats may be impaired thrombus neovascularization. Thrombus neovascularization has been tightly associated with DVT resolution in both humans and rodent models.[15,24,25] Neutrophils pro-

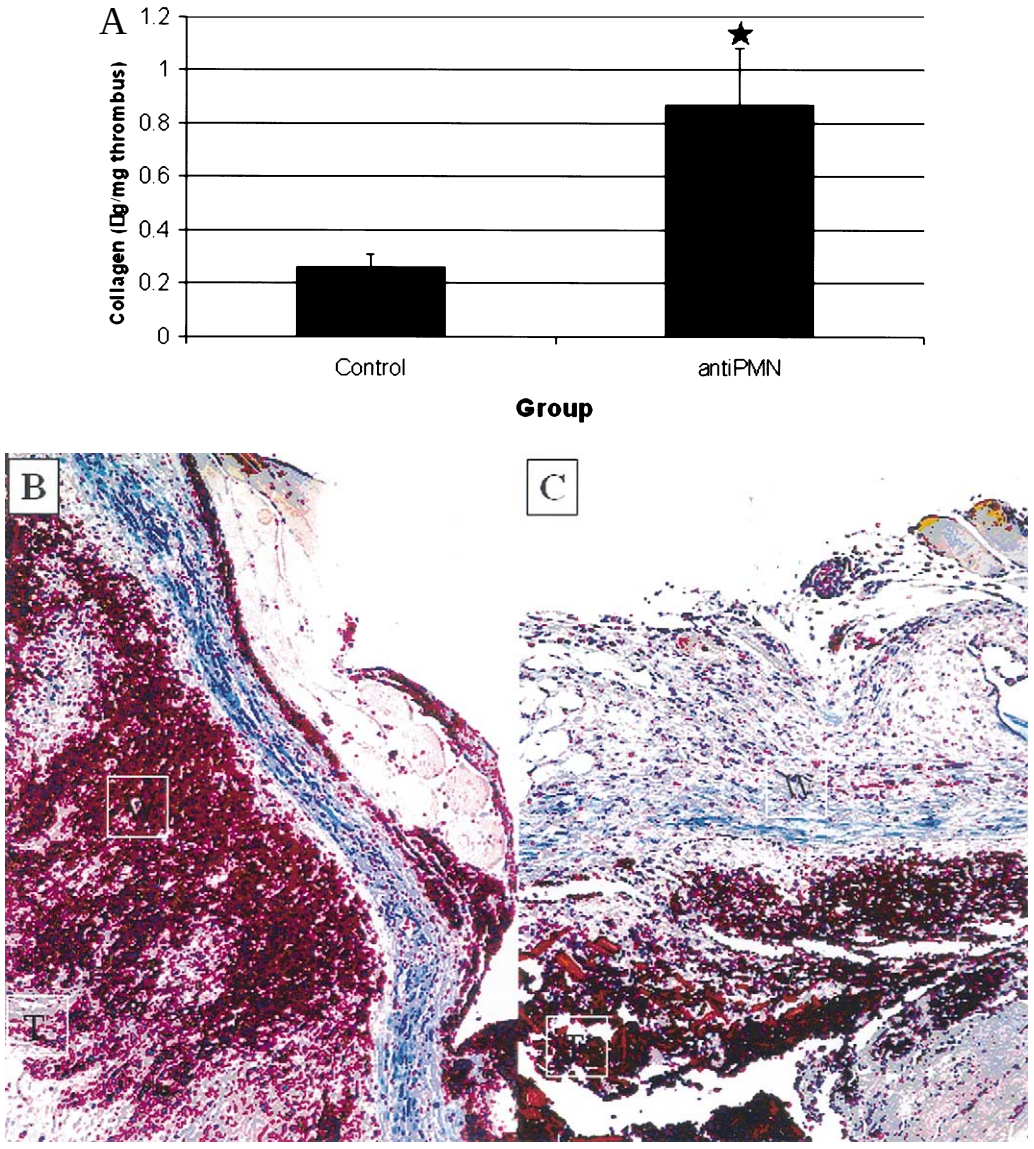

Fig 6. A, Sircol collagen assay. There was significantly more collagen (types I to III) remaining in the thrombi of neutropenic rats compared with controls at 7 days. This is reflected in Trichrome-stained section (100×) from (**B**) control and (**C**) neutropenic rat thrombi. Note increased fibrosis within the thrombus proper as well as the vein wall in the neutropenic IVC section, stained whitish-blue. Clot is red color. T = thrombus. W = vein wall.

duce angiogenic mediators, such as vascular endothelial growth factor,[26] and a proangiogenic milieu is found in resolving thrombi.[27] Previous studies by our group have shown that the proinflammatory cytokine IL-8 accelerates DVT resolution and was associated with increased neovascular channel formation, as estimated by thrombus colloidal carbon staining and laminin immunohistochemical staining of neovascular channels.[15,25] It is likely that the thrombus neovascularization that forms is most important for allowing influx of later inflammatory cells such as monocytes rather than providing for substantial prograde venous flow. Venography is not sensitive enough to delineate microvascular channels but did show that venous collateral formation was not dependent on PMNs, although these channels represent small lumbar vessels that dilate and do not require de novo formation from biochemical mediators. Indeed, neutropenic rats had larger and more persistent collaterals, which shows that the thrombus burden was physiologically significant as well. Further studies on the determinants of the angiogenic aspects of thrombus resolution are likely to clarify the role(s) of PMNs in this process.

An interesting finding was the increased thrombus fibrosis in the neutropenic rats. This suggests that the PMN

is important for the normal breakdown of early wound collagen in the thrombus, similar to a nonluminal wound. In all acute processes, type III collagen is laid down as a provisional matrix for influx of structural cells and leukocytes.[13] Neutrophils release collagenases, such as matrix metalloproteinases 8 and 9, that actively degrade collagen in addition to their activity of plasminogen activation.[28] Neutrophils also secrete elastase with collagenolytic activity.[23] Interestingly, our prior work with IL-8 supplementation showed increased thrombus fibrosis, but the assay used in that study was not as sensitive as the one in the current study. In addition, the possibility that stimulating PMNs with an exogenous chemokine may increase reactive oxygen species production exists.[7,15]

As the most common white blood cells and the principal cellular effector of the early inflammatory response, PMNs are essential to resolution of many physiologic insults. Neutrophil depletion has been studied in a wide variety of models of injury, including angioplasty-induced arterial injury,[29] abdominal wounds,[30] cerebral infection,[19] and reperfusion injury,[31] to name a few. Most of these studies have indicated that PMNs cause damage in these modeled clinical settings, but it really depends on the clinical situation to determine when PMNs are detrimental. Neutropenia is a common clinical sequelae of disease and certain therapeutic medications. PMNs are often depleted in patients with cancer, and neutrophil chemotaxis has been shown to be impaired in vitro in patients with shock.[29] In the setting of malignancy, the incidence of DVT is increased as well and Lin et al have shown a significant association between increasing number of leukopenic episodes and recurrence of DVT in cancer patients.[2] Although several other factors affect the incidence of DVT in cancer patients, including the type of cancer and the chemotherapeutic regimen, this correlation suggests that in the dynamic setting of thrombus formation and resolution, leukopenia may decrease the host's natural thrombus resolution capability. Thus, asymptomatic DVT that normally would have resolved have a higher likelihood of becoming symptomatic in these patients.

This work has shown that PMNs contribute to early DVT resolution, which suggests an important role for a proinflammatory environment. Further experimental work will define the mechanisms of the PMNs and monocytes on in vivo thrombolytic and angiogenic activity, to allow potential pharmacologic targets for therapy without the hazards of anticoagulation.

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