

# CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

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## AN ANTI-INFLAMMATORY PROPERTY OF APROTININ DETECTED AT THE LEVEL OF LEUKOCYTE EXTRAVASATION

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**Background:** Aprotinin is a serine protease inhibitor used extensively in cardiac operations to reduce postoperative bleeding. It has also been used in trials aimed at reducing the systemic inflammatory response to cardiopulmonary bypass. It remains unclear whether the anti-inflammatory action of aprotinin is related to its general ability to suppress leukocyte activation or whether aprotinin can exercise effects during the leukocyte-endothelial cell adhesion cascade.

**Methods:** We used intravital microscopy to study the 3 main stages of the adhesion cascade (leukocyte rolling, firm adhesion, and extravasation) within the mesenteric microcirculation of rats. This *in vivo* technique allows leukocyte recruitment to be viewed directly through the transparent mesentery of anesthetized animals.

**Results:** Aprotinin, given by continuous infusion at a clinically relevant dose, exerted no effect on the rolling or firm adhesion responses toward local chemoattractant *N*-formyl-methyl-leucyl-phenylalanine but significantly inhibited extravasation of leukocytes (73% at 40 minutes,  $P = .04$ ) into surrounding tissues. In parallel *in vitro* experiments, aprotinin (used at 200, 800, and 1600 kIU/mL) dose dependently inhibited neutrophil transmigration through cultured endothelial cells in response to 3 different chemoattractants: *N*-formyl-methyl-leucyl-phenylalanine ( $P < .001$  at 800 and 1600 kIU/mL), interleukin 8 ( $P < .05$  at 200 kIU/mL and  $P < .001$  at 800 and 1600 kIU/mL), and platelet-activating factor ( $P < .05$  at 1600 kIU/mL).

**Conclusions:** Our studies have therefore revealed a novel anti-inflammatory mechanism of aprotinin operating at the level of leukocyte extravasation. These findings may be relevant in the prevention of systemic inflammation after cardiopulmonary bypass through the use of protease inhibitors. (J Thorac Cardiovasc Surg 2000;120:361-9)

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Aproinin (Trasylol) is a nonspecific serine protease inhibitor that has been used extensively in cardiac operations since its efficacy in reducing postoperative bleeding was discovered in the mid-1980s.<sup>1</sup> It possesses broad hemostatic properties that are mediated by

blocking pathways of complement activation and fibrinolysis, as well as by inhibiting the action of proteinases, such as trypsin, plasmin, and kallikrein.<sup>2</sup> Although used mainly for its hemostatic effects, aprotinin is also thought to modify the inflammatory response to major

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operations through a general ability to inhibit neutrophil activation.<sup>3,4</sup>

The inflammatory response is characterized by the adhesion of circulating leukocytes to microvascular endothelium and subsequent extravasation into tissues. Three distinct phases have been described: (1) initial attachment and rolling of leukocytes along the vessel wall, (2) firm adhesion, and (3) transmigration into the extravascular tissue.<sup>5</sup> In broad terms the first phase is mediated by the selectin family of adhesion molecules (E-, L-, and P-selectin), and the second is mediated by the integrin family (leukocyte function-associated antigen-1, Mac-1, and very late activation antigen-4). Least is known about the third step, but several immunoglobulin supergene family members are involved on the endothelial side, including intracellular adhesion molecule and CD31,<sup>6</sup> whereas integrins and proteases have been implicated on the leukocyte side.<sup>7</sup>

The systemic inflammatory response syndrome (SIRS) is a clinical syndrome that may arise as a result of various insults, such as major trauma, a major operation, sepsis, or cardiopulmonary bypass (CPB). SIRS is associated with leukocyte sequestration into tissues, and the organs affected and severity differ quite widely among patients.<sup>8</sup> In its extreme form SIRS can lead to multiple organ failure that often includes adult respiratory distress syndrome, a condition associated with massive leukocyte infiltration in the lung and high mortality.<sup>9,10</sup> Little direct evidence exists regarding adhesion molecule expression involved in the sequestration of leukocytes during SIRS. The significance of various inflammatory mediators and leukocyte chemoattractants in acute lung injury has been investigated in animal models of CPB-related injury and also in trials involving human subjects. Analysis of the pathophysiologic mechanism involved in these studies suggests that activation of complement components and subsequent neutrophil and monocyte activation are followed by pulmonary endothelial injury, production of interleukin (IL) 8 and platelet-activating factor (PAF), and migration of neutrophils and monocytes into the alveoli.<sup>11-13</sup>

We conducted this study to investigate whether aprotinin could exert anti-inflammatory effects at the level of leukocyte interactions with the vessel wall. Intravital microscopy was used to investigate leukocyte responses within rat mesentery, an experimental approach that has been instrumental in defining and resolving the 3 main phases of leukocyte recruitment during inflammation *in vivo*.<sup>5,14</sup> Using this technique, we have previously shown roles for endogenously generated PAF and the adhesion molecule CD31 in leukocyte extravasation through rat mesenteric venules.<sup>6,15</sup> Here we report that continuous infusion of aprotinin at a clinically relevant

dose exerted no effect on rolling or firm adhesion events but selectively inhibited the leukocyte extravasation step in response to the chemoattractant *N*-formyl-methyl-leucyl-phenylalanine (fMLP). Furthermore, aprotinin dose dependently inhibited fMLP-, IL-8-, or PAF-induced neutrophil transmigration across human umbilical vein endothelial cell (HUVEC) monolayers *in vitro*.

## Methods

**Materials.** Aprotinin, fMLP, PAF, and endothelial cell growth factor supplement were obtained from Sigma (Dorset, United Kingdom). Recombinant 72 aa human IL-8 was purchased from Peprotech (London, United Kingdom).

**Intravital microscopy.** Male Sprague-Dawley rats (250-300 g) were purchased from Harlan-Olac (Bicester, United Kingdom) and prepared for intravital microscopy, as previously described.<sup>7</sup> In brief, rats were anesthetized intravenously with 20 mg/kg sodium pentobarbital (Sagatal; May and Baker Ltd, Dagenham, United Kingdom), and anesthesia was maintained intravenously with 20 mg · kg<sup>-1</sup> · h<sup>-1</sup> of sodium pentobarbital. Anesthetized animals were placed on a heated stage at 37°C, and a 1- to 2-cm midline abdominal incision was made to expose the small intestine. A segment of the terminal ileum was carefully exteriorized and placed over a transparent glass window. The exposed tissue was continuously superfused with Tyrode's salt solution (Sigma Chemical Co) maintained at 37°C and bubbled with 5% carbon dioxide in air. The whole preparation was mounted onto the stage of a Zeiss Axioskop microscope (Carl Zeiss Ltd, Welwyn Garden City, United Kingdom), and the mesenteric microcirculation was viewed by means of high-magnification water-dipping objectives. Images were monitored with a color, chilled, video camera (C5810, Hamamatsu Photonics United Kingdom Ltd, Enfield, United Kingdom) mounted on the microscope and recorded by an S-VHS videocassette recorder. Leukocyte responses were quantified in a randomly selected 100- $\mu$ m segment of postcapillary venule with a 25- to 40- $\mu$ m diameter. Rolling cells were defined as cells traveling visibly slower than the flowing erythrocytes past a fixed point. At each time point the number of rolling cells was counted for 5 minutes, and the mean value was taken. Firmly adherent cells were defined as those that remained stationary for at least 30 seconds within the 100- $\mu$ m vessel segment. Leukocyte extravasation was quantified by counting the number of cells that had emigrated up to 50  $\mu$ m away from the vessel wall.

Baseline readings of rolling, firm adhesion, and extravasation were made before infusion with aprotinin or saline control. To design an aprotinin dose regimen in rats that did not exceed levels achieved in clinical practice, we took into account the fact that aprotinin was removed by renal clearance at a glomerular filtration rate ( $\sim$ 3-4 mL · kg<sup>-1</sup> · min<sup>-1</sup> for a 300-g rat) that was higher compared with that of the human patient ( $\sim$ 1.5 mL · kg<sup>-1</sup> · min<sup>-1</sup> for a 70-kg human being).<sup>16</sup> Aprotinin was given as a loading dose of 40,000 kIU/kg fol-

lowed by an infusion of  $20,000 \text{ kIU} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ . The total dose given to each rat over the course of an experiment was approximately  $60,000 \text{ kIU/kg}$ , which compares with the total dose given to a patient throughout a CPB operation (between  $60,000$ - $120,000 \text{ kIU/kg}$ ).<sup>17</sup> Fifteen minutes after recording baseline readings, fMLP was added to the Tyrode superfusant (at a final fMLP concentration of  $10^{-7} \text{ mol/L}$ ), and further measurements were made over the next 60 minutes. Data are represented as means  $\pm$  SEM from 5 rats in each treatment group. Differential white cell counts were obtained before and after aprotinin infusion (3 rats per group) by diluting blood 1:10 in Kimura stain<sup>18</sup> and counting cells on a hemocytometer.

**Neutrophil isolation from human venous blood.** Peripheral venous blood from 5 healthy nonsmoking human donors was anticoagulated immediately by using 2 mL of sodium citrate 3.8% (Pharma Hameln, Hameln, Germany) per 25 mL of blood and placed in aliquots in polypropylene tubes. After centrifugation at 3000 rpm for 10 minutes, the plasma was removed, and cells were mixed with 6 mL of 6% high-molecular-weight dextran (Dextran T500; Pharmacia Biotech, Uppsala, Sweden) and 20 mL of phosphate-buffered saline solution. After 25 minutes to sediment red cells, the leukocyte-rich upper layer was harvested, centrifuged at 1200 rpm for 5 minutes, resuspended in 2 mL of autologous plasma, and layered onto a 2-step (74% and 50%) Percoll (Pharmacia) gradient. After centrifugation at 1300 rpm for 10 minutes, neutrophils were collected from the top of the 74% interface, washed twice in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2% fetal calf serum, and counted.

**Neutrophil transmigration through HUVEC monolayers.** HUVECs were obtained from umbilical cords by using collagenase type II (Boehringer) digestion, as previously described.<sup>19</sup> HUVECs were maintained on 1% (vol/vol) gelatin-coated (Sigma) tissue-culture flasks in growth medium consisting of M199 supplemented with 20% heat-inactivated ( $56^\circ\text{C}$  for 30 minutes) fetal calf serum (HyClone Laboratories Inc, Salt Lake City, Utah), 100 IU/mL penicillin, 100  $\mu\text{g/mL}$  streptomycin,  $2 \times 10^{-6} \text{ mol/L}$  L-glutamine, 10 U/mL heparin, and 30  $\mu\text{g/mL}$  endothelial cell growth factor (Sigma). HUVEC monolayers were established 1 day before transmigration experiments on cell culture inserts (3.0- $\mu\text{m}$  pore size filters, Falcon; Becton-Dickinson, Franklin Lakes, NJ) coated with 50  $\mu\text{g/mL}$  fibronectin. After excess fibronectin had been rinsed off, inserts were placed into 24-well plates (Nunc; Nalge Nunc International, Denmark), and  $1 \times 10^5$  HUVECs were added to each insert. After overnight incubation, endothelial cells were preincubated for 60 minutes with aprotinin at 3 doses (200, 800, or 1600 kIU/mL). Filters were washed with RPMI medium, and subsequently, RPMI containing fMLP ( $10^{-9} \text{ mol/L}$ ), IL-8 ( $10^{-9} \text{ mol/L}$ ), or PAF ( $10^{-6} \text{ mol/L}$ ) was added to the lower chamber. Neutrophils were preincubated with aprotinin or medium for 15 minutes and added at  $1 \times 10^6$  to the upper surface of filter inserts. Neutrophil transmigration was measured after 60 minutes by removing filters and counting cells that had migrated into the lower chamber. So that the independent

effects of aprotinin on endothelial cells or neutrophils could be studied, transmigration experiments were also performed by preincubating HUVECs only or neutrophils only with aprotinin (1600 kIU/mL) before chemotactic responses to fMLP or IL-8.

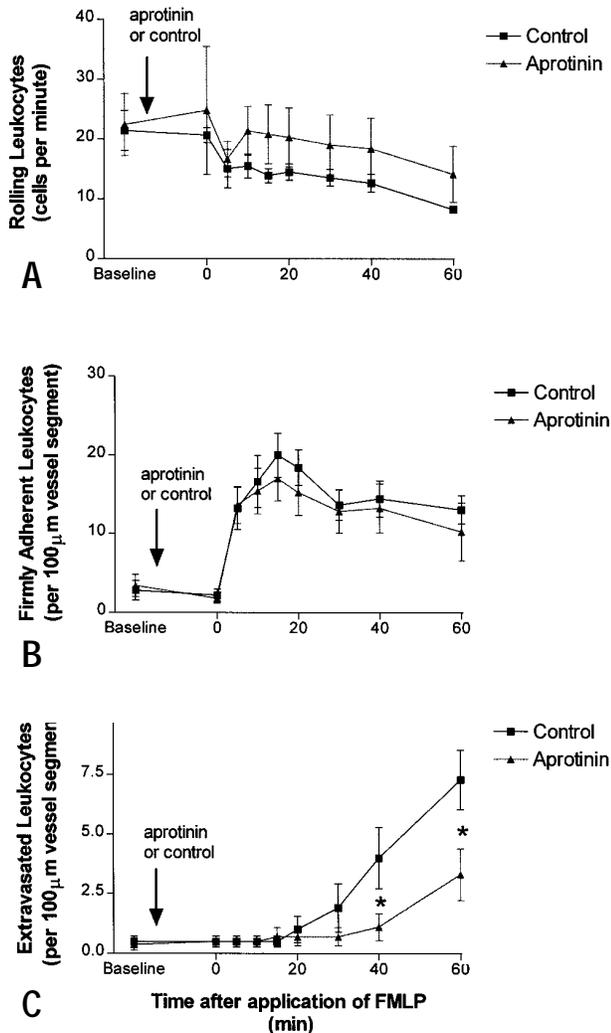
**Myeloperoxidase assay.** Peripheral venous blood collected as described above was preincubated with aprotinin at 3 doses (200, 800, and 1600 kIU/mL) for 15 minutes at room temperature on a rotating wheel, followed by a further 60-minute incubation in the presence of fMLP ( $10^{-7} \text{ mol/L}$ ). Plasma was obtained from whole blood at the end of the experimental treatment period by means of centrifugation at 3000 rpm for 10 minutes and stored at  $-70^\circ\text{C}$  until analysis. The enzyme-linked immunosay for myeloperoxidase was performed with 100  $\mu\text{L}$  of human plasma in a 96-well microplate format according to the manufacturer's instructions (OXIS International Inc, Portland, Ore). Plasma samples and myeloperoxidase standards were assayed in duplicate, and concentrations were calculated from a standard curve. Results are expressed as means  $\pm$  SEM from 5 donors.

**Statistical analysis.** Data from intravital studies were analyzed by means of the Mann-Whitney *U* test for the comparison of nonparametric data (GraphPad Prism Software, Inc, San Diego, Calif). Neutrophil transmigration and myeloperoxidase enzyme-linked immunosorbent assay data were analyzed by a 1-way analysis of variance with a Newman-Keuls post-test (GraphPad Prism).

## Results

**Aprotinin inhibits leukocyte extravasation in vivo.** In exteriorized rat mesenteric tissues, there was a basal level of leukocyte rolling ranging between 5 and 33 cells/min, a low level of firmly adherent leukocytes within venules, and a small number of leukocytes in the extravascular tissue (Figs 1 and 2). After the topical application of the chemoattractant fMLP ( $10^{-7} \text{ mol/L}$ ), although there was no change in the rolling flux response, leukocyte adhesion and extravasation were significantly increased in a time-dependent manner (Fig 1).

Aprotinin was infused into rats by using a protocol equivalent to the high-dose regimen used in CPB, which aims to achieve a constant concentration of 200 kIU/mL in the circulation throughout the duration of the procedure.<sup>17</sup> Each experiment was approximately 90 minutes in duration, consisting of 15 minutes to exteriorize the mesentery, 15 minutes to pretreat with aprotinin or saline control, and 60 minutes to observe leukocyte trafficking in response to fMLP. In fMLP-treated venules aprotinin exerted no significant effect on leukocyte rolling (Fig 1, A) or firm adhesion (Fig 1, B) but significantly inhibited extravasation at 40 minutes ( $P < .05$ ) and 60 minutes ( $P < .05$ ; Fig 1, C). Fig 2 depicts a representative venule from each treatment



**Fig 1.** Aprotinin inhibits leukocyte extravasation but not leukocyte rolling or firm adhesion within rat mesenteric venules. Leukocyte endothelial cell responses were quantified during a 60-minute time course after fMLP application ( $10^{-7}$  mol/L) in randomly selected 100- $\mu$ m mesenteric vessel segments from each animal. Aprotinin did not affect leukocyte rolling (A) or firm adhesion (B) but significantly attenuated transmigration through the vessel wall at 40 and 60 minutes after topical application of fMLP (C). Results are expressed as means  $\pm$  SEM ( $n = 5$  animals). \* $P < .05$ .

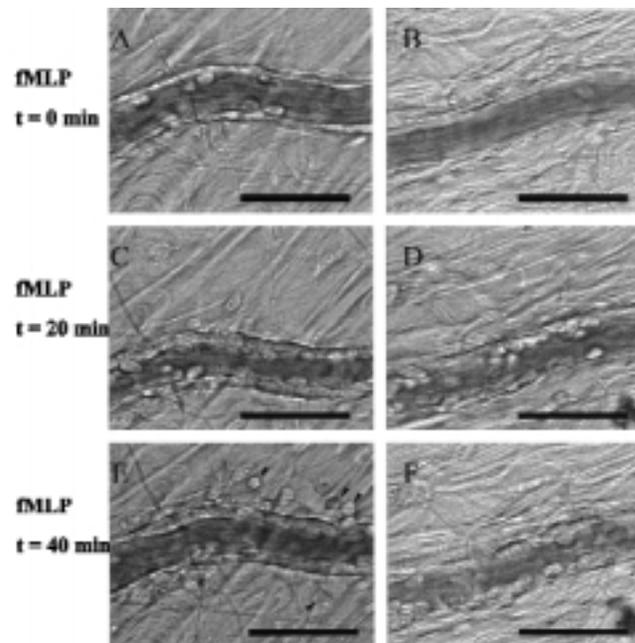
group, which demonstrates that emigration of leukocytes up to 50  $\mu$ m from the vessel wall was attenuated by aprotinin (Fig 2, F) compared with the control group (Fig 2, E). This effect was not caused by possible alterations in circulating white cell counts because there was no significant difference between the aprotinin and saline treatment groups for these variables (Table I). Furthermore, it is highly unlikely that the observed effects were caused by alterations in microhemody-

amic variables because aprotinin had no effect on leukocyte rolling, a response that is highly susceptible to changes in hemodynamic factors.<sup>20</sup>

**Aprotinin inhibits neutrophil extravasation in vitro.** Because protease inhibitors have been previously shown to inhibit leukocyte transmigration through cultured endothelial cells,<sup>21</sup> in parallel with the in vivo investigations, we investigated the effect of aprotinin on leukocyte extravasation in vitro. In these experiments purified human neutrophils were added to confluent lawns of HUVECs grown on transwell filter inserts (3- $\mu$ m pore size), and endothelial transmigration was measured in response to chemoattractant added to the lower chamber. A portion (7.3%) of cells added to the upper chamber spontaneously transmigrated into the lower chamber, and this was increased to 25.7% by the addition of chemoattractant fMLP ( $10^{-9}$  mol/L) to the lower chamber (Fig 3, A). Aprotinin significantly inhibited the extravasation response to the level of background at 800 kIU/mL ( $P < .001$ ) and below levels of background at 1600 kIU/mL ( $P < .001$ ). IL-8 and PAF are chemoattractants that are thought to be involved in the recruitment of neutrophils in the alveolar space in acute lung injury.<sup>11,13</sup> IL-8 ( $10^{-9}$  mol/L) was the most potent chemotactic stimulus used in these studies, promoting 46.1% of cells to transmigrate, but this response was also significantly attenuated by aprotinin at 200 kIU/mL ( $P < .05$ ), 800 kIU/mL ( $P < .001$ ), and 1600 kIU/mL ( $P = .001$ ). Similarly, transmigration in response to PAF ( $10^{-6}$  mol/L) was significantly inhibited to the level of background by aprotinin at 1600 kIU/mL ( $P < .05$ ; Fig 3, C). Thus, aprotinin dose dependently and significantly inhibited in vitro transmigration of neutrophils in response to the chemoattractants fMLP, IL-8, and PAF.

To address which cell type was targeted by aprotinin in the in vitro transmigration assay, we compared the effect of preincubating neutrophils or HUVECs alone with the effect of treating both cells in combination, as in the preceding experiments. This analysis showed that preincubation of either neutrophils (15 minutes) or HUVECs (60 minutes) alone incompletely inhibited chemotactic responses to fMLP but that combined treatment of both cell types reduced transmigration to background levels (Fig 4, A). Similar additive responses were observed by using IL-8 as a chemotactic stimulus (Fig 4, B). Thus, we have demonstrated that the inhibition of transendothelial migration by aprotinin is likely to involve a complex mechanism of action, involving potential target molecules on both the transmigrating leukocyte and endothelial cell.

**Effect of aprotinin on neutrophil myeloperoxidase secretion.** Neutrophil migration into the subendothelial



**Fig 2.** Effect of aprotinin on leukocyte endothelial cell responses viewed by intravital microscopy. Photomicrographs of representative segments of postcapillary venule in rat mesentery are shown, as viewed by intravital microscopy. Both saline control (A) and aprotinin-treated animals (B) exhibited a basal leukocyte rolling flux before application of fMLP. Twenty minutes after fMLP, firm adhesion was evident in both control (C) and aprotinin-treated animals (D). Forty minutes after fMLP, leukocytes that had transmigrated were visible around the postcapillary venule (arrows) in control (E) but not aprotinin-treated animals (F) (bar = 50  $\mu$ m).

**Table I.** Effect of aprotinin infusion on peripheral blood counts

	Before infusion			After infusion		
	Total	Mononuclear cells	Granulocytes	Total	Mononuclear cells	Granulocytes
Saline						
Experiment 1	$4.33 \times 10^6$	$3.02 \times 10^6$	$1.31 \times 10^6$	$2.64 \times 10^6$	$1.64 \times 10^6$	$1.00 \times 10^6$
Experiment 2	$5.92 \times 10^6$	$4.60 \times 10^6$	$1.31 \times 10^6$	$3.26 \times 10^6$	$2.53 \times 10^6$	$7.30 \times 10^5$
Experiment 3	$5.54 \times 10^6$	$3.92 \times 10^6$	$1.62 \times 10^6$	$3.14 \times 10^6$	$2.02 \times 10^6$	$1.12 \times 10^6$
Aprotinin*						
Experiment 1	$4.77 \times 10^6$	$3.76 \times 10^6$	$1.01 \times 10^6$	$2.70 \times 10^6$	$1.46 \times 10^6$	$1.24 \times 10^6$
Experiment 2	$5.50 \times 10^6$	$4.42 \times 10^6$	$1.08 \times 10^6$	$2.88 \times 10^6$	$2.28 \times 10^6$	$6.00 \times 10^5$
Experiment 3	$6.46 \times 10^6$	$5.06 \times 10^6$	$1.40 \times 10^6$	$2.21 \times 10^6$	$1.20 \times 10^6$	$1.01 \times 10^6$

Peripheral venous blood was collected from rats (n = 3) before and after saline or aprotinin infusion. Peripheral blood was differentially stained by using Kimura's stain,<sup>28</sup> and total white cells, mononuclear cells, and granulocytes were counted on a hemocytometer. Results are expressed as cells per milliliter.

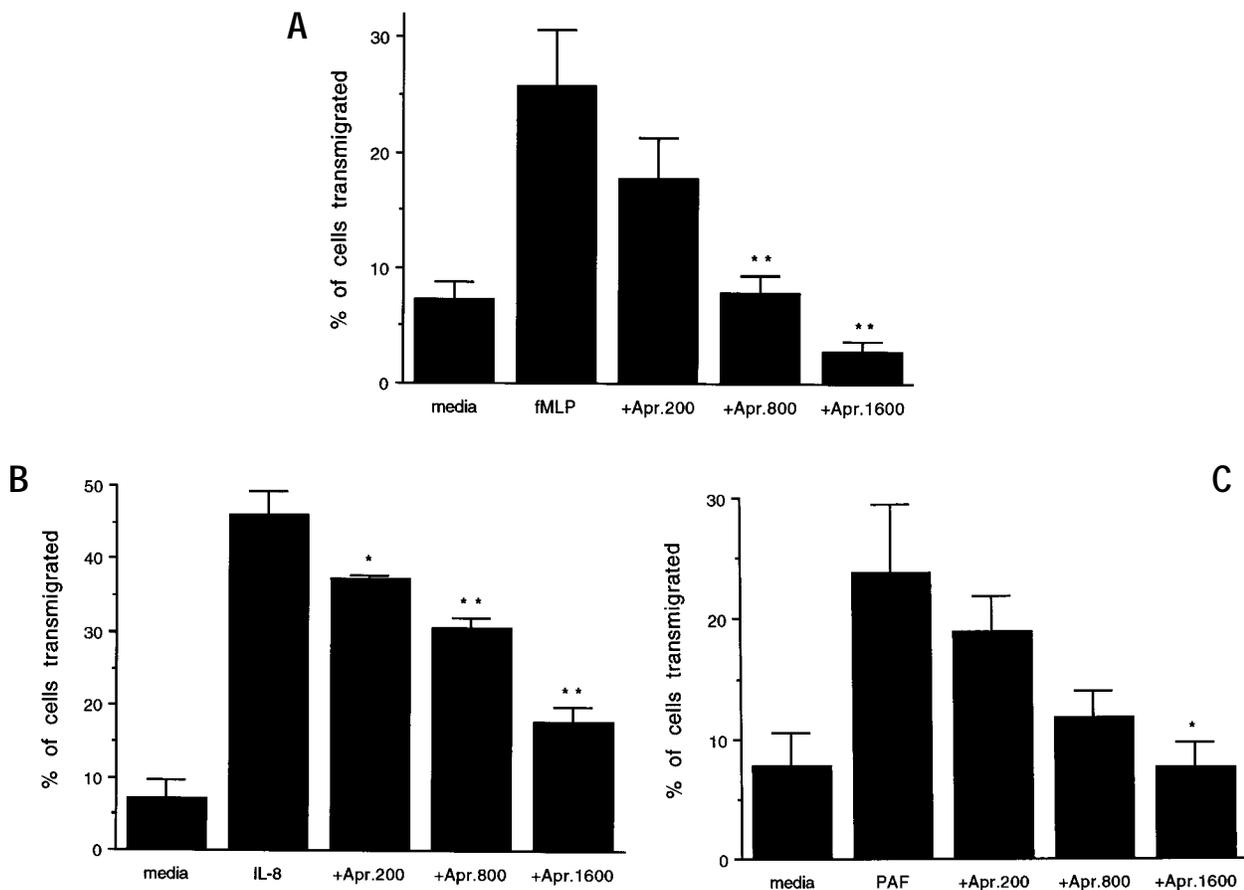
\*None of the aprotinin treatment groups differed significantly from the saline control groups.

space, recruitment into alveoli, and activation with release of granular contents are regarded as decisive steps in acute lung injury.<sup>22</sup> We wished therefore to examine whether aprotinin could inhibit myeloperoxidase secretion from neutrophils. This was studied by measuring the release of myeloperoxidase from human neutrophils in whole blood in response to fMLP. Aprotinin dose dependently inhibited myeloperoxidase secretion, which diminished progressively to media levels at 800 kIU/mL

( $P < .05$ ; Fig 5). We have therefore demonstrated that aprotinin, in addition to inhibiting extravasation, can also inhibit secretion of myeloperoxidase from neutrophils. In a clinical setting these properties of aprotinin may combine to limit lung injury after CPB.

### Discussion

We have shown that aprotinin can inhibit leukocyte extravasation at a clinically relevant dose both in vivo and

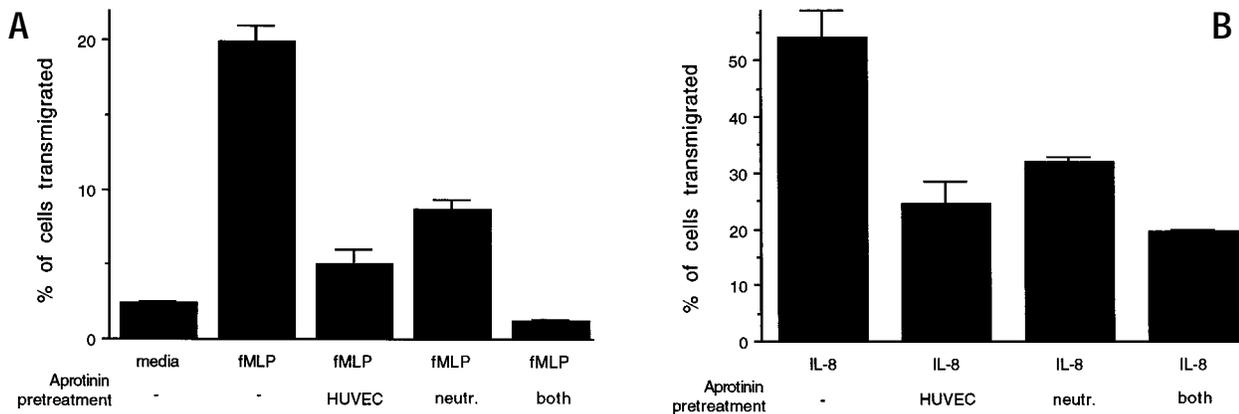


**Fig 3.** Aprotinin inhibits neutrophil transmigration across HUVECs in response to fMLP, IL-8, and PAF. Freshly isolated neutrophils from human venous blood were allowed to transmigrate in the presence of the indicated doses of aprotinin for 60 minutes through cultured endothelial cells on filters (3- $\mu$ m pore size) in response to the following chemoattractants in the lower chamber: fMLP ( $10^{-9}$  mol/L, **A**), IL-8 ( $10^{-9}$  mol/L, **B**), and PAF ( $10^{-6}$  mol/L, **C**). Neutrophils and HUVECs were pretreated with aprotinin for 15 and 60 minutes, respectively, before the addition of chemoattractant in the lower chamber. Results are expressed as mean percentage of cells transmigrated  $\pm$  SEM from 5 experiments by using separate HUVEC isolates and peripheral blood donors. \* $P < .05$ ; \*\* $P < .001$  from chemoattractant without aprotinin.

in vitro. This was demonstrated by results of intravital microscopy studies in rat mesentery in response to the chemoattractant fMLP, as well as in transmigration assays through HUVECs in response to the neutrophil chemoattractants fMLP, IL-8, and PAF. Our experiments comprise the first direct demonstration that a step in the leukocyte adhesion cascade can be inhibited by aprotinin and may explain previous observations of reduced leukocyte recruitment in the presence of protease inhibitors within the lung after CPB or in experimental models of pneumonitis in sheep.<sup>23,24</sup>

The strength of the in vivo work lies in the ability of intravital microscopy to provide a direct measure of sep-

arate stages of the leukocyte-endothelial cell adhesion cascade. With this technique, we were able to demonstrate that intravenous aprotinin at a dose equivalent to that used in cardiac operations (see the "Methods" section) could exert a specific inhibitory effect on leukocyte extravasation induced by fMLP without concomitant effects on leukocyte rolling or firm adhesion. Results in the animal model, which used fMLP as an acute topical chemoattractant, were supported by in vitro transmigration experiments and extended to two further chemoattractants, IL-8 and PAF, which are relevant to lung injury.<sup>11,13</sup> Chemotactic responses to all 3 stimuli were approximately 20% to 30% diminished in



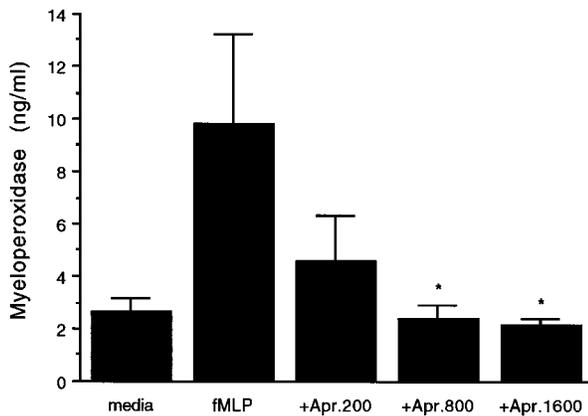
**Fig 4.** Aprotinin can target neutrophils or endothelial cells in isolation. Chemotactic responses to fMLP (A) and IL-8 (B) were carried out as described in the legend to Fig 3, pretreating either neutrophils (15 minutes) or HUVECs (60 minutes) alone or in combination with aprotinin at a dose of 1600 kIU/mL. Results shown are from representative experiments expressed as mean percentage of cells transmigrated  $\pm$  SD. All assays were performed in duplicate.

the *in vitro* studies at a clinically relevant 200 kIU/mL dose of aprotinin, whereas higher doses virtually abolished transmigration. The *in vitro* model also demonstrated that aprotinin could target either neutrophils or endothelial cells independently. Both experimental models are representative of a short-term response to local chemoattractant. We therefore consider it unlikely that aprotinin exerted its effects by suppressing endothelial cell activation, although it remains possible that chemoattractants may have primed endothelial cell function, as has been previously reported by the careful analysis of cell shape change by electron microscopy in the *in vivo* responses to fMLP.<sup>25</sup> We are currently extending the study of aprotinin to longer term models of leukocyte extravasation that involve the explicit prior activation of endothelial cells.

In a physiologic context the possible mechanism of action of aprotinin falls into two main categories: (1) direct effects on proteases involved in the transmigration process or (2) indirect effects brought about through diminished neutrophil activation. With respect to the first possibility, the elastase and cathepsin families of serine proteases have been implicated in both the extravasation and tissue migration processes and may therefore represent direct targets for aprotinin.<sup>7,21</sup> Another family of proteases involved in cell migration are the metalloproteinases.<sup>7</sup> Metalloproteinase-2 (gelatinase A), for example, has been found at the leading edge of migrating cells in the form of a covalent complex bound to integrin  $\alpha_v\beta_3$ .<sup>26</sup> A possible indirect effect of aprotinin on metalloproteinases may occur as a result of its well-documented inhibition of plasmin.

Recent animal studies involving mice deficient in plasminogen and components of the plasminogen-activating system have shown that plasmin may play a critical role in the proteolytic activation of latent metalloproteinase proenzymes, a necessary event during the migration of monocytes across the elastic lamina.<sup>27,28</sup>

The second major possible mechanism of action, that aprotinin may block extravasation as a consequence of diminished leukocyte activation, is also plausible. In this respect aprotinin has been previously shown to block the activation-dependent secretion of elastase from neutrophils and cell-surface expression of Mac-1, events that could both play a role in leukocyte extravasation.<sup>3,4</sup> In the present study we have also shown that aprotinin can block secretion of the neutrophil granule component myeloperoxidase, and in the clinical setting this effect may act synergistically with diminished extravasation to limit leukocyte damage to inflamed organs. Candidate-signaling molecules capable of transducing proteolytic signals into the cell include the protease-activated receptor family.<sup>29</sup> Although these are poorly studied on leukocytes, we have observed that protease-activated receptor-1-dependent responses in platelets are blocked by aprotinin (Poullis M, Manning R, Laffan M, Haskard DO, Taylor KM, Landis RC; see page 370), therefore supporting a possible role for protease-activated receptors as targets for the anti-inflammatory action of aprotinin. Taken together, the above lines of evidence suggest that the molecular targets of aprotinin in the extravasation process are likely to be complex, consisting of multiple targets both in the soluble phase and associated with



**Fig 5.** Aprotinin inhibits myeloperoxidase secretion from human neutrophils. Peripheral venous blood was incubated at room temperature for 60 minutes with fMLP ( $10^{-7}$  mol/L) in the presence and absence of aprotinin at the indicated doses. Plasma was collected, and immunoreactive myeloperoxidase was measured by means of enzyme-linked immunosorbent assay. All samples were measured in duplicate. Results are expressed as mean myeloperoxidase (in nanograms per milliliter)  $\pm$  SEM ( $n = 5$  donors). \* $P < .001$  from fMLP without aprotinin.

the cell surface of endothelial cells, migrating leukocytes, or both.

The question arises as to whether other proteases with greater selectivity than aprotinin may represent an alternative treatment strategy to combat the systemic inflammatory response after a major operation. One of the advantages of using aprotinin in trials aimed at reducing systemic inflammation is that unlike other agents, aprotinin is already in clinical use (Trasylol) and has sufficiently low toxicity when administered in the approved dose. Another advantage is that its broad spectrum of action may provide anti-inflammatory protection in addition to proven hemostatic benefits. Activation of complement, secretion of inflammatory cytokines, and neutrophil activation are all important steps in the inflammatory cascade that are targeted by aprotinin. The use of sensitive markers of leukocyte activation suggest that a majority of patients may experience at least a measurable degree of neutrophil activation during CPB,<sup>4,30</sup> although certain groups of patients may be primed to react more severely on the basis of emergency operation, poor cardiac output, or sepsis.<sup>8,10,30</sup> Our findings may be of particular relevance to such patient groups at risk of SIRS because the leukocyte extravasation step might be more effectively treated

through higher doses of aprotinin or, in due course, other classes of protease inhibitors with greater specific activity or longer circulating half-life than aprotinin.

In conclusion, our study has revealed that leukocyte extravasation, a necessary step in the inflammatory cascade, is inhibited by aprotinin. Because this effect was achieved with a clinically relevant dose of aprotinin, it is likely that aprotinin exerts an anti-inflammatory effect by reducing leukocyte extravasation when used in patients undergoing CPB. Additional patient benefits may be achieved in the future through the identification of protease inhibitors with more specific anti-inflammatory activities.

#### REFERENCES

1. Royston D, Bidstrup BP, Taylor KM, Salford RN. Effect of aprotinin on need for blood transfusions after repeat open heart surgery. *Lancet* 1987;2:1289-91.
2. Taylor KM. Effect of aprotinin on blood loss and blood use after cardiopulmonary bypass. In: Pifarre R, editor. Anticoagulation, hemostasis, and blood preservation in cardiovascular surgery. Philadelphia: Hanley & Belfus; 1993. p. 132.
3. Wachtfogel YT, Kucich U, Hack CE, Gluszko P, Niewiarowski S, Colman RW, et al. Aprotinin inhibits the contact, neutrophil, and platelet activation systems during simulated extracorporeal perfusion. *J Thorac Cardiovasc Surg* 1993;106:1-10.
4. Hill GE, Alonso A, Spurzem JR, Stammers AH, Robbins RA. Aprotinin and methylprednisolone equally blunt cardiopulmonary bypass-induced inflammation in humans. *J Thorac Cardiovasc Surg* 1995;110:1658-62.
5. Frenette PS, Wagner DD. Adhesion molecules—Part II: blood vessels and blood cells. *N Engl J Med* 1996;335:43-5.
6. Wakelin MW, Sanz M-J, Dewar A, Albelda SM, Larkin SW, Boughton-Smith N, et al. An anti-platelet-endothelial cell adhesion molecule-1 antibody inhibits leukocyte extravasation from mesenteric microvessels in vivo by blocking the passage through the basement membrane. *J Exp Med* 1996;184:229-39.
7. Bianchi E, Bender JR, Blasi F, Pardi R. Through and beyond the wall: late steps in leukocyte transendothelial migration. *Immunol Today* 1997;18:586-91.
8. Asimakopoulos G, Taylor KM, Smith PL, Ratnatunga CP. Prevalence of acute respiratory distress syndrome after cardiac surgery. *J Thorac Cardiovasc Surg* 1999;117:620-1.
9. Milberg JA, Davis DR, Steinberg KP, Hudson LD. Improved survival of patients with acute respiratory distress syndrome (ARDS): 1983-1993. *JAMA* 1995;273:306-9.
10. Christenson JT, Aeberhard JM, Badel P, Pepcak F, Maurice J, Simonet F, et al. Adult respiratory distress syndrome after cardiac surgery. *Cardiovasc Surg* 1996;4:15-21.
11. Jorens PG, Van Damme J, De Backer W, Bossaert L, De Jongh RF, Herman AG, et al. Interleukin-8 in the bronchoalveolar lavage fluid from patients with the adult respiratory distress syndrome (ARDS) and patients at risk for ARDS. *Cytokine* 1992;4:592-7.
12. Dreyer WJ, Michael LH, Millman EE, Berens KL, Geske RS. Neutrophil sequestration and pulmonary dysfunction in a canine

- model of open heart surgery with cardiopulmonary bypass: evidence for a CD18-dependent mechanism. *Circulation* 1995; 92:2276-83.
13. Miotla JM, Jeffery PK, Hellewell PG. Platelet-activating factor plays a pivotal role in the induction of experimental lung injury. *Am J Respir Cell Mol Biol* 1998;18:197-204.
  14. Ley K. Molecular mechanisms of leukocyte recruitment in the inflammatory process. *Cardiovasc Res* 1996;32:733-42.
  15. Nourshargh S, Larkin SW, Das A, Williams TJ. Interleukin-1-induced leukocyte extravasation across rat mesenteric microvessels is mediated by platelet-activating factor. *Blood* 1995;85:2553-8.
  16. Treeck B, Aukland K. Effect of L-NAME on glomerular filtration rate in deep and superficial layers of rat kidneys. *Am J Physiol* 1997;272:F312-8.
  17. Royston D. Controversies in the practical use of aprotinin. In: Pifarré R, editor. *Anticoagulation, hemostasis, and blood preservation in cardiovascular surgery*. Philadelphia: Hanley & Belfus; 1993. p. 150.
  18. Kimura I, Moritani Y, Tanizaki Y. Basophils in bronchial asthma with reference to reagin-type allergy. *Clin Allergy* 1973;3:195-202.
  19. Wellicome SM, Thornhill MH, Pitzalis C, Thomas DS, Lanchbury JS, Panayi GS, et al. A monoclonal antibody that detects a novel antigen on endothelial cells that is induced by tumor necrosis factor, IL-1, or lipopolysaccharide. *J Immunol* 1990;144:2558-65.
  20. Firrell JC, Lipowsky HH. Leukocyte margination and deformation in mesenteric venules of rat. *Am J Physiol* 1989;256:H1667-74.
  21. Cepinskas G, Noseworthy R, Kvietys PR. Transendothelial neutrophil migration: role of neutrophil-derived proteases and relationship to transendothelial protein movement. *Circ Res* 1997;81:618-26.
  22. Eppinger MJ, Deeb GM, Bolling SF, Ward PA. Mediators of ischemia-reperfusion of rat lung. *Am J Pathol* 1997;150:1773-84.
  23. Hill GE, Pohorecki R, Alonso A, Rennard SI, Robbins RA. Aprotinin reduces interleukin-8 production and lung neutrophil accumulation after cardiopulmonary bypass. *Anesth Analg* 1996;83:696-700.
  24. O'Riordan TG, Otero R, Mao Y, Lauredo I, Abraham WM. Elastase contributes to antigen-induced mucociliary dysfunction in ovine airways. *Am J Respir Crit Care Med* 1997;155:1522-8.
  25. Feng D, Nagy JA, Pyne K, Dvorak HF, Dvorak AM. Neutrophils emigrate from venules by a transendothelial cell pathway in response to fMLP. *J Exp Med* 1998;187:903-15.
  26. Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, et al. Localisation of matrix metalloproteinase (MMP)-2 to the surface of invasive cells by interaction with integrin alpha V beta 3. *Cell* 1996;85:683-93.
  27. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaître V, Tipping P, et al. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* 1997;17:439-44.
  28. Moons L, Shi C, Ploplis V, Plow E, Haber E, Collen D, et al. Reduced transplant arteriosclerosis in plasminogen-deficient mice. *J Clin Invest* 1998;102:1788-97.
  29. Coughlin SR. How the protease thrombin talks to cells. *Proc Natl Acad Sci U S A* 1999;96:11023-7.
  30. Asimakopoulos G, Kohn A, Haskard DO, Landis RC, Taylor KM. Effect of aprotinin on  $\beta$ 1 and  $\beta$ 2 leukocyte integrins in patients undergoing cardiopulmonary bypass: a randomized study. *Ann Thorac Surg*. In press.

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