Leukocyte activation does not mediate myocardial leukocyte retention during endotoxemia in rabbits

CHRISTOPHER M. GODDARD, BETTY Y. POON, M. EMILIA KLUT, BARRY R. WIGGS, STEPHAN F. VANEEDEN, JAMES C. HOGG, AND KEITH R. WALLEY
Pulmonary Research Laboratory, St. Paul’s Hospital, University of British Columbia, Vancouver, British Columbia, Canada V6Z 1Y6

Goddard, Christopher M., Betty Y. Poon, M. Emilia Klut, Barry R. Wiggs, Stephan F. vanEeden, James C. Hogg, and Keith R. Walley. Leukocyte activation does not mediate myocardial leukocyte retention during endotoxemia in rabbits. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1548–H1557, 1998.—Our goal was to determine whether coronary leukocyte retention after endotoxin infusion was due primarily to leukocyte activation. Leukocytes were activated by infusion of endotoxin into 12 blood donor rabbits. Separately, 12 isolated rabbit hearts were perfused with blood from an endotoxemic support rabbit to expose coronary endothelium to an inflammatory stimulus. During an infusion of 20 ml of donor blood into the isolated heart, the coronary transit time of leukocytes was determined by deconvolution of multiple measurements of injectate and collected leukocyte concentrations. With no leukocyte activation or inflammatory stimulation of endothelium, leukocyte transit time was 9.2 ± 3.5 s, and 11.6 ± 4.1 × 10³ leukocytes were retained in the coronary circulation. Leukocyte activation alone did not alter transit time (9.8 ± 3.2 s) or retention (9.3 ± 4.6 × 10³ leukocytes). Inflammatory stimulation of endothelium with and without leukocyte activation increased transit time (18.0 ± 3.6 and 18.9 ± 3.8 s, respectively; P < 0.05) and retention (24.8 ± 8.4 and 25.3 ± 6.8 × 10³ leukocytes, respectively; P < 0.05) to the same extent. Differential counts showed that neutrophils (but not lymphocytes) were slowed and retained. Inflammatory stimulation of endothelium caused coronary capillary endothelial swelling and pseudopod formation. Thus increased coronary neutrophil transit time and retention are due to structural changes of coronary endothelium caused by inflammatory response (9, 24). Whether acute leukocyte retention in the myocardium is dependent on leukocyte activation, endothelial cell structural changes, or a combination of both is not known.

To address this issue, we utilized an isolated, support rabbit heart model of acute endotoxemia that has previously been shown to replicate both the myocardial depression and leukocyte retention (9) observed during whole animal sepsis (8). By measuring multiple consecutive leukocyte concentrations in blood flowing into [I(t)] and out of [O(t)] the coronary circulation at the time of a step change in arterial leukocyte concentration, we determined the average leukocyte transit time as the mean of the transfer function [T(t)] deconvoluted from [I(t)] and [O(t)] (22) such that [T(t) · T(t) = O(t)] (Fig. 1). By measuring differential leukocyte fractions of leukocyte distributions, we were able to determine the relative contribution of neutrophils (polymorphonuclear neutrophils PMN]) and lymphocytes to the total leukocyte coronary transit time.

METHODS

This study was approved by the University of British Columbia animal care committee.

Surgical preparation of support rabbit. Twenty-four 2.5 ± 0.5-kg female New Zealand White rabbits were anesthetized initially with a mixture of ketamine (40 mg/kg; MTC Pharmaceuticals, Cambridge, ON, Canada) and xylazine (5 mg/kg; Chemagro, Etobicoke, ON, Canada). To maintain deep surgical anesthesia for the duration of the experiment, α-chloralose (55 mg/kg iv; Sigma, St. Louis, MO) and urethan (12 mg/kg iv; Sigma) were then injected via the left marginal ear vein. The criteria for deep surgical anesthesia were the absence of lacrimation and no change in heart rate or blood pressure after a painful stimulus applied to a hind toe. Depth of anesthesia was tested hourly and before any intervention. We found that supplementary anesthesia was not required. A midline ventral incision was made in the neck, and a tracheostomy tube was inserted. Rabbits were ventilated with room air and supplemental O₂ with the use of a Harvard ventilator (Harvard Apparatus Canada, Saint-Laurent, PQ, Canada) to maintain PO₂ at ~400 mmHg and PCO₂ at ~35 mmHg. Polyethylene catheters (ID 1.67 mm, OD 2.42 mm; Intramedic, Becton-Dickinson, Parsippany, NJ) were inserted into the right carotid artery and the left external jugular vein to perfuse and drain the extracorporeal circuit for the isolated heart. A three-way stopcock allowed the infusion of fluids and
preparation of support rabbit. Hearts were rapidly excised via a midline sternotomy and affixed by the aorta to the perfusion column. A Thebesian drain was not inserted because of the very small proportion of the total myocardial blood flow (~5%) accounted for by the Thebesian circulation. The absence of a Thebesian drain also prevented contamination of collected fractions by blood potentially flowing at a rate different from that of the main coronary circulation. The isolated heart was paced at 150 beats/min. The isolated heart was then allowed to equilibrate for 15 min to ensure stability of rhythm and total coronary blood flow.

Langendorff column and extracorporeal circuit. A modified Langendorff column and extracorporeal circuit was utilized as previously described (Fig. 2) (9). Arterial blood from the support rabbit was pumped via the carotid arterial catheter with the use of a roller pump (Masterflex, Cole-Parmer Instrument, Chicago, IL) through two leukocyte filters (Pall Biomedical Products, East Hills, NY) placed in series to exclude all support rabbit leukocytes from the circulation of the isolated heart (<1.0 × 10^5 leukocytes/l). Blood flowed from an open 75-mmHg perfusion column that was attached to the proximal aorta of the isolated heart via a plastic cannula (ID 3.2 mm, OD 4.8 mm) to perfuse the isolated heart. Blood overflowing from the perfusion column and venous blood from the isolated heart were pumped with the use of a roller pump through a 40-µm blood filter (SQ40S Blood Transfusion Filter, Pall Biomedical Products) back to the support rabbit via the jugular venous catheter. Total coronary blood flow was continuously measured with the use of an ultrasonic flow transducer (Transonic Systems, Ithaca, NY) inserted in the aortic cannula just above the aortic valve.

Leukocytes for perfusion. Twenty-four 2.0 ± 0.5-kg rabbits were anesthetized and surgically prepared in a fashion identical to that of the support rabbits. The isolation or labeling of leukocytes invariably causes their activation. Therefore, to prevent random leukocyte activation in some experimental groups, we chose to collect leukocytes in whole blood. Rabbits received either endotoxin (100 µg/kg iv; Escherichia coli O111:B4, Sigma), to activate circulating leukocytes, or vehicle. Arterial blood was withdrawn at 2.0 ml/min from all rabbits with the use of a syringe pump (Harvard Apparatus, Holliston, MA) into a sealed, heparinized (500 µl, 1:1,000) 35-ml syringe that was gently agitated continuously to prevent settling of formed blood elements. In all experiments leukocyte-containing blood was used within 30 s of collection.

Protocol. All experiments were conducted as follows. Isolated hearts were perfused with leukocyte-depleted carotid arterial blood from their support rabbits for 2 h after the start of an infusion of vehicle or endotoxin (100 µg/kg iv over 30 min). After this 2-h period, support rabbit perfusion was stopped and the isolated hearts were perfused with 20 ml of leukocyte-containing blood from a single blood donor rabbit at identical flow rate and perfusion pressure. After the leukocyte-containing blood had been infused, the leukocyte-depleted support rabbit perfusion circuit was restored.

Experimental groups were defined based on whether the perfusing leukocytes or the isolated heart was activated by endotoxin. Four experimental groups were studied (Table 1). In group 1 (n = 6), the control, blood donor, and support rabbits each received vehicle only so that neither the perfusing leukocytes nor the isolated heart was activated. In group 2 (n = 6), the blood donor rabbit received endotoxin and the support rabbit received vehicle so that perfusing leukocytes were activated and the isolated heart was not. In group 3 (n = 6), the blood donor rabbit received endotoxin and the support rabbit received vehicle so that perfusing leukocytes were not activated, whereas the isolated heart was activated. In
group 4 (n = 6), the blood donor and support rabbits both received endotoxin so that both the perfusing leukocytes and the isolated heart were activated.

A fraction collector was used to collect coronary venous effluent in continuous 0.5-ml aliquots in individual glass collection vials containing 40 µl of EDTA solution. Six baseline samples of venous effluent during support rabbit perfusion were collected. Venous aliquot sampling was continued during and after leukocyte infusion until leukocyte concentrations in venous blood had decreased back to baseline to allow leukocytes still transiting the heart to exit the system.

At the completion of the protocol, the support rabbit circulation was interrupted and oxygenated normal saline at 37°C was infused at identical flow and pressure until the venous effluent was free of red blood cells (≈2 min). Glutaraldehyde 2.5% in phosphate-buffered saline was then added to the perfusion circuit, and the heart was perfusion fixed for 15 min. The aorta of the isolated heart was then ligated, and the heart was immersed in a large volume of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3, room temperature) to fix for an additional 48 h.

Measurement of leukocyte activation. The infusion of endotoxin in vivo causes the activation of circulating leukocytes (2, 38). To confirm that leukocyte activation occurred in this experiment, we quantified baseline expression of CD18 (38) on PMN with the use of immunofluorescent flow cytometric analysis of blood samples taken from donor rabbits before operation (baseline). After infusion of endotoxin or vehicle into the donor rabbit, a blood sample was taken directly from the syringe sample to determine the degree of activation of
leukocytes before infusion through the isolated heart. Ten sequential blood samples were then obtained from the blood aliquots after transit through the isolated heart, and the resultant mean value for CD18 activation was used to describe the activation of blood after perfusion through the isolated heart. Leukocytes were labeled in whole blood. The technique for preparing cells for cytometric analysis is described elsewhere (8) (mouse anti-rabbit CD18 antibody 60.3 was used courtesy of Dr. E. C. Butcher, Stanford University School of Medicine, Stanford, CA). Flow cytometry was performed on the specimens within 24 h (Profile EPIC II, Coulter Electronics, Hialeah, FL). Analysis gates for the neutrophil subset of leukocytes were established using the distinctive forward and side scatter profiles (representative data shown in Fig. 3). Data were expressed as mean fluorescence intensity (MFI) and normalized to the MFI of leukocytes in blood from donor rabbits before operation (baseline).

Calculation of total and differential leukocyte content. Measurements of total leukocyte concentration (S880 Automated Hematology Analyzer, Coulter Counter, Coulter Electronics) were made on each 0.5-ml aliquot of blood collected after transit through the coronary circulation of the isolated heart and on a sample aliquot from the syringe before and after infusion of endotoxin or vehicle. Similar samples were analyzed from each blood donor rabbit before infusion of endotoxin or vehicle. To determine the relative content of various leukocyte populations, a thin blood smear was prepared from each aliquot and from the syringe sample. Blood smears were stained with Quick Wright's stain (Camco Quick Stain II, Cambridge Diagnostic Products, Fort Lauderdale, FL). The differential leukocyte count was quantified by counting 100 cells/smear at ×40 magnification. Multiplication of the individual leukocyte fraction by the total number of leukocytes in the same aliquot yielded the total number of each leukocyte fraction in that aliquot. In this way the total number of lymphocytes and PMN in each aliquot was quantified. We found that, after activation by endotoxin, sample aliquots of blood contained only negligible numbers of monocytes, so those cells were not quantified.

Calculation of transit times. Leukocyte number was plotted versus time for the infused blood and for blood exiting the heart (Fig. 1). Our goal was to determine the transfer function T(t) such that, for the input distribution function I(t) and output distribution O(t),

\[ \frac{d}{dt} \int T(t) dt = O(t) \]

Because differentiation is the inverse of integration, it follows that, when I(t) is a step function, the transit time distribution is simply given by the time derivative of O(t). Numerical differentiation amplifies noise in the measured output leukocyte-concentration function. Therefore, to per-

Table 1. Experimental groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Heart</th>
<th>Leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>Nonactivated; support rabbit received vehicle only</td>
<td>Nonactivated; donor rabbit received vehicle only</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Nonactivated; support rabbit received vehicle only</td>
<td>Activated; donor rabbit received endotoxin before blood harvest</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>Activated; support rabbit received endotoxin 2 h before leukocyte infusion</td>
<td>Nonactivated; donor rabbit received vehicle only</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>Activated; support rabbit received endotoxin 2 h before leukocyte infusion</td>
<td>Activated; donor rabbit received endotoxin before blood harvest</td>
</tr>
</tbody>
</table>

Endotoxin was infused at 100 µg/kg iv over 30 min; n = no. of rabbits.
form this differentiation, we first fit the output distribution with a three-parameter logistic model given as \( O(t) = \frac{a}{1 + \exp(\beta - yt)} \) (32). We found that this distribution very closely fit the measured output distribution of leukocyte concentrations \( r^2 > 0.95 \). The mean of the time derivative of this distribution was calculated to determine the mean leukocyte transit time for that experiment. This approach permits recovery and quantitation of the entire injectate sample.

To determine the transit time of the apparatus, five calibration experiments were performed in identical fashion, but with exclusion of the isolated heart, over a range of flow rates. Regression analysis on the relationship between flow and perfusion apparatus transit time gave an \( r^2 \) value of 0.98, indicating that apparatus transit time could be closely predicted by the flow rate. Therefore, the flow-dependent transit time of the perfusion apparatus was subtracted from the total transit time for each experimental measurement to yield mean coronary transit time of leukocytes.

Calculation of the individual coronary transit times of lymphocytes and neutrophils was performed by plotting the mean values for the number of lymphocytes and neutrophils per aliquot from all experiments in each group versus time. Mean lymphocyte and neutrophil coronary transit times were then calculated for each group as described above.

Calculation of leukocyte retention. Transit time measurements reflect slowing of leukocytes that transit the coronary circulation. We also measured retention of leukocytes that enter but do not exit the coronary circulation, because retention of leukocytes appears to be mediated by mechanisms different from that of slowing (15, 34). The total number of leukocytes entering the heart was calculated as the total volume infused (20 ml for each heart) multiplied by the total leukocyte concentration of the sample (\( \times 10^6 \) cells/ml) to yield total number of leukocytes infused. The total number of leukocytes exiting the heart was calculated as the volume of all vials containing leukocytes (0.5 ml for each vial) multiplied by the total leukocyte concentration in each vial. The total number of leukocytes retained in the heart was calculated as the difference between the total number of leukocytes entering and exiting the heart. The fraction of total leukocytes retained was calculated as the total number retained divided by the total number entering the heart.

The numbers of lymphocytes and neutrophils in each blood aliquot were determined as the percentages of each cell type multiplied by the total number of leukocytes in that aliquot to determine the relative retention of lymphocytes and neutrophils.

Morphometric analysis of leukocyte retention and endothelial activation. To verify leukocyte retention by the coronary circulation, morphometric analysis of the fixed isolated hearts was performed (1, 16). Fixed left ventricles were sliced in a transaxial orientation into adjacent 3-μm tissue disks. Random tissue disks were selected for analysis. Tissue disks were dehydrated and embedded in paraffin blocks. A 3-μm tissue section was cut from each block and stained with hematoxylin and eosin. Samples were examined at \( \times 100 \) magnification. Random fields were assessed by enumerating all leukocytes within the field. Ten random fields were assessed for each tissue section. The mean density of leukocytes was calculated as the number of leukocytes counted per tissue section divided by 10 fields.

To verify the presence of structural changes indicating endothelial activation, transmission electron microscopy (TEM) was performed. Random tissue samples \((-1 \text{ mm}^3)\) were excised from the glutaraldehyde-fixed left ventricles. After specimens were washed with buffer, they were postfixed in 1% osmium tetroxide, washed, dehydrated in a graded ethanol series, and embedded in LR White (Sigma). Sections were obtained on an RMC MT 6000-XL ultramicrotome and examined on a Philips transmission electron microscope at \( \times 400 \) magnification.

Data analysis. A two-way ANOVA was used to test for differences among groups in leukocyte transit time and leukocyte retention. If a significant difference was found, unpaired t-tests, corrected for multiple comparisons using a sequentially rejective Bonferroni test procedure, were used. Values obtained for MFI of the donor blood sample were compared among groups and with the resultant MFI of the output samples using a two-way ANOVA. \( P < 0.05 \) was chosen as statistically significant. Data are expressed as means \( \pm \) SD throughout.

RESULTS

There was no significant increase in activation of neutrophils, as measured by MFI, in infused blood from donor rabbits receiving vehicle only (groups 1 and 3) (Fig. 4). This indicates that the collection and handling of the leukocyte-containing blood and the injection of vehicle did not lead to activation of neutrophils. There was a significant increase of MFI of neutrophils in infused blood from blood donor rabbits receiving endotoxin (groups 2 and 4), indicating activation of these neutrophils. There was no significant activation of neutrophils in blood exiting hearts whose blood donor and support rabbits had received vehicle only (group 1), indicating that nonspecific factors related to tubing, connectors, admixture of blood types, or the collection of samples did not lead to activation of leukocytes. In group 3, neutrophils in blood exiting hearts showed a small but significant increase in MFI, indicating that neutrophils could be activated by transiting activated hearts. There was no significant change in MFI of
activated neutrophils transiting the coronary circulation of hearts whose support rabbits had received vehicle or endotoxin (groups 2 and 4, respectively).

Activation of leukocytes did not alter the total leukocyte coronary transit time in groups with nonactivated hearts (group 1 vs. group 2) or the longer total leukocyte coronary transit time in groups with activated hearts (group 3 vs. group 4) (Fig. 5), indicating that total leukocyte coronary transit time was independent of the state of activation of leukocytes entering the coronary circulation. However, total leukocyte coronary transit time approximately doubled in the groups with activated coronary endothelium (groups 3 and 4) versus the groups with nonactivated coronary endothelium (groups 1 and 2) (P < 0.05; Fig. 5). Neither leukocyte activation nor coronary endothelial activation altered lymphocyte coronary transit time (Fig. 6). Thus the observed increase in total leukocyte coronary transit time of groups 3 and 4 was accounted for by the increase in neutrophil coronary transit time (Fig. 6).

In addition to these differences in transit times of leukocytes that transit the coronary circulation, we also observed differences in retention of leukocytes that enter but do not exit the coronary circulation. Leukocyte activation did not alter the absolute (Table 2) or percent leukocyte retention (Fig. 7) by the coronary circulation in groups with nonactivated hearts (group 1 vs. group 2). Leukocyte activation did not alter the absolute leukocyte retention by the coronary circulation in groups with activated hearts (group 3 vs. group 4), and the trend toward increased percent leukocyte retention in group 4 compared with that in group 3 (Fig. 7) was not statistically significant. These results indicate that retention of leukocytes was not affected by the state of activation of leukocytes entering the coronary circulation. However, there was a significant increase in the total number of leukocytes retained by the coronary circulation when the coronary endothelium was activated (groups 3 and 4 vs. groups 1 and 2) (Table 2 and Fig. 7). There was an apparent trend toward increased retention of lymphocytes in groups with activated coronary endothelium (groups 3 and 4). However, this apparent retention was not statistically sig-

<table>
<thead>
<tr>
<th>Table 2. Coronary leukocyte retention and coronary blood flow</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td><strong>Nonactivated endothelium</strong></td>
</tr>
<tr>
<td>1 Tot</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2 Tot</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Activated endothelium</strong></td>
</tr>
<tr>
<td>3 Tot</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4 Tot</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Data are means ± SD; n = 6 rabbits/group. Tot, total leukocytes; PMN, polymorphonuclear neutrophils; Lym, lymphocytes. *Significantly different (P < 0.05) from control (group 1). Values for PMN and Lym do not summate to Tot due to presence of other leukocyte fractions in whole blood.
significant with respect to absolute (Table 2) or percent retention of lymphocytes (Fig. 8) by any group, because the variability in retention of the lymphocyte subset was high in relation to the mean difference. There was significant absolute (Table 2) and percent retention of neutrophils by groups 3 and 4 versus groups 1 and 2 (Fig. 8), indicating that the increased total leukocyte retention observed in groups 3 and 4 was accounted for by neutrophils.

There was no difference in total coronary blood flow among groups (Table 2). There was no change in coronary perfusion pressure from the preset column pressure of 75 mmHg at any time during the experiment.

Morphometric quantification of myocardial leukocytes (Table 3) supported these observations of leukocyte retention by showing no significant effect of leukocyte activation on density of leukocytes (leukocytes/high-power field). However, groups with activated endothelium (groups 3 and 4) had significantly increased myocardial leukocyte density compared with groups with nonactivated endothelium (groups 1 and 2), consistent with the observation of increased leukocyte retention. The majority of retained leukocytes were neutrophils. Leukocytes were retained primarily within coronary capillaries at this time point.

Activation of coronary endothelium was verified by TEM morphometric analysis. Figure 9A demonstrates the normal appearance of coronary endothelium derived from hearts not exposed to endotoxin. Electron micrographs in Fig. 9, B–D, are derived from ventricles exposed to endotoxin. These hearts demonstrate structural changes that are the hallmark of endotoxin-induced activation. Figure 9B demonstrates endothelial cell swelling and intraluminal pseudopod formation that partially obstruct the capillary lumen. Figure 9C demonstrates endothelial cell membrane changes in apposition to a PMN. In Fig. 9D a PMN lies in close apposition to the endothelial cell membrane, forming a closed space referred to as a lacuna. A PMN granule has been discharged into this space.

DISCUSSION

These results suggest that slowing and retention of leukocytes in the coronary microcirculation that is observed in animal models of sepsis or acute endotoxemia (2, 8, 9, 10) is primarily due to activation of the coronary endothelium or other effects of the inflammatory response occurring within coronary capillaries. Leukocyte activation, which leads to increased neutrophil rigidity and increased expression of adhesion proteins (15), appears to be much less important in causing neutrophil slowing and retention. Interest-

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Leukocytes/HPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>(0.17 \pm 0.04)</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>(0.47 \pm 0.11)</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>(1.00 \pm 0.38^*)</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>(1.32 \pm 0.65^*)</td>
</tr>
</tbody>
</table>

Data are means ± SD (n = 6 rabbits/group) for total leukocytes per high-power field (HPF; \(\times 100\) magnification) of left ventricular sections. The majority of leukocytes counted were PMN. *Significantly different (\(P < 0.05\)) from control (group 1).
ingly, lymphocyte transit time is not significantly affected by either coronary endothelial activation or leukocyte activation. Thus neutrophils are the leukocytes that have increased transit times and increased retention in this model of acute endotoxemia. Several mechanisms may contribute to neutrophil slowing and retention in the coronary circulation, including neutrophil-related factors, changes in microcirculatory dynamics, endothelium-specific factors, or other effects of the inflammatory response occurring within coronary capillaries.

A number of neutrophil-related factors may contribute to neutrophil slowing and retention (29) during sepsis. Tumor necrosis factor (TNF-α) and other proinflammatory mediators can suppress neutrophil chemotaxis, increase adhesion of neutrophils to gelatin, increase surface CD11b expression, and increase rigidity of the neutrophil membrane (34). Decreased fluidity of leukocytes may impede or prevent capillary transit to postcapillary venules, leading to entrapment. This “stiffening” effect of neutrophil activation on neutrophil retention in the lung has been well described (15). Thus the anticipated effect of leukocyte activation would be to increase the slowing and retention of neutrophils within the coronary capillaries, because the average neutrophil diameter of 6.6 ± 0.6 µm (5) is greater than the average coronary capillary diameter of 5.6 ± 1.3 µm (11). In the present study there is an apparent trend toward increased leukocyte retention after activation (group 1 vs. group 3; Table 3); however, the observed difference did not achieve statistical significance. This suggests that rheologically mediated events were relatively insignificant to retention in this model. Receptor-mediated adhesion of leukocytes to vascular endothelial cells has been demonstrated (7). Similar to previous observations (38), we found increased expression of the integrin receptor CD18 on activated neutrophils. However, neutrophil CD18 upregulation alone in this model did not lead to increased neutrophil retention.

Although we did not observe differences in total coronary blood flow or total coronary perfusion pressure, we have previously found that endotoxin infusion results in increased heterogeneity of microcirculatory blood flow (13). Changes in microcirculatory dynamics due to local mediators, such as nitric oxide (19) or TNF-α (36), or alterations in sympathetic neurotransmission (39) leading to a reduction in coronary perfusion pressure or blood flow conceivably could lead to increased retention of neutrophils. In addition, it is possible that alterations in microcirculatory dynamics due to changes in red blood cell deformability (3), platelet aggregation (14), or other factors not affecting measures of bulk flow may have caused local capillary hypoperfusion with resultant leukocyte retention. Fur-
 thermore, areas of the coronary microcirculation with reduced flow may permit settling of leukocytes on the endothelial surface and thereby facilitate bonding of adhesion molecules.

A number of endothelium-specific factors may contribute to the retention of leukocytes. These factors comprise both functional and structural changes and are collectively referred to as activation. During sepsis, endotoxin activates endothelial cells via direct (e.g., soluble CD14-endotoxin complexes) and indirect pathways (e.g., monocyte-bound CD14-endotoxin complexes) (31), with the indirect pathway being quantitatively more significant. Monocytes bound to endotoxin via membrane-associated CD14 receptors are stimulated to secrete TNF-α and interleukin (IL)-1 (12). These proinflammatory cytokines may stimulate further proinflammatory mediator production, including IL-1 and IL-6, by endothelial cells (4, 28). Endothelial cell adhesion molecule expression is complex and requires a progression of pathological stimuli to manifest a proadhesive surface (27, 35). In addition, increased production of tissue factor by activated endothelial cells leads to the local deposition of thrombin (33), which may further disrupt local microcirculatory dynamics, leading to leukocyte retention. Local thrombin deposition may lead to increased production of platelet-activating factor by endothelial cells (37), which may prime neutrophil responses to activating stimuli. Proinflammatory cytokines also induce chemokine expression so that the activated heart expresses leukocyte chemotactic signals (25). Abnormal endothelial cell membrane transport function occurring during sepsis (18) leads to endothelial cell swelling. Endothelial cell swelling (21) in association with intracapillary fibrin deposition (33) may cause or contribute to the observed increased heterogeneity of microvascular blood flow (17) during sepsis and passive entrapment of leukocytes in nonflowing microvascular segments. Further investigation is required to determine which of these many potential factors are most important in accounting for our observations.

Analysis of the leukocyte effluent from experiments in which only the heart was activated (group 3) demonstrated an increase in CD18 MFI after transit of the coronary circulation, consistent with activation of leukocytes within the heart itself. This and other experimental evidence supports the speculation that the evolution of the sepsis syndrome involves early proinflammatory cytokine expression in the blood, leading to activation of endothelium and other cells within various organs. The affected tissue then becomes the source of mediators capable of causing leukocyte retention and activation. Juxtaposition of activated leukocytes and activated endothelial cells may be essential for leukocyte-mediated endothelial cell damage (37). Thus activated endothelium in combination with the retained leukocytes and proinflammatory mediators may cause local damage. In the heart, morphometric evidence of damage is closely associated with alteration in organ function (26).

Although endothelial activation appears to play an important role in leukocyte slowing and retention (groups 3 and 4), the inflammatory mediator environment within the capillaries (e.g., cytokines, platelet-activating factors, soluble CD14 receptors, etc.) activates leukocytes (group 3) and, therefore, may also contribute to leukocyte slowing and retention independent of endothelial activation. For example, chemokines released by the cardiac myocytes (25) activate neutrophils and may establish a chemotactic gradient that contributes to neutrophil retention. Other inflammatory mediators expressed in higher concentrations within coronary capillaries may also contribute in a similar way.

In summary, this study demonstrates that, during sepsis, retention of leukocytes is relatively independent of leukocyte activation. Activation of the coronary endothelium may be a crucial factor in the retention of leukocytes by the coronary circulation.

This work was supported by the Medical Research Council of Canada. C. M. Goddard is a fellow of the Heart and Stroke Foundation of British Columbia and Yukon. B. R. Wiggs is a research scholar of the Medical Research Council of Canada and the British Columbia Lung Association. K. R. Walley is a British Columbia Lung/St. Paul’s Hospital Foundation Scientist.

Address for reprint requests: K. R. Walley, Pulmonary Research Laboratory, St. Paul’s Hospital, 1081 Burrard St., Vancouver, BC, Canada V6Z 1Y6.

Received 29 August 1997; accepted in final form 19 July 1998.

REFERENCES