Aspects of leucocyte and fat filtration during cardiac surgery

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CHAPTER 4

FILTRATION OF ACTIVATED GRANULOCYTES DURING CARDIOPULMONARY BYPASS SURGERY: A MORPHOLOGIC AND IMMUNOLOGIC STUDY TO CHARACTERIZE THE TRAPPED LEUCOCYTES.

J.J.J. Smit, A.J. de Vries, Y.J. Gu, W. van Oeveren

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ABSTRACT

Cardiopulmonary bypass surgery induces an inflammatory reaction among others by granulocytes. Leucocyte filtration has been shown to reduce the postoperative morbidity mediated by activated granulocytes. However, little is known about the mechanism of filter-leucocyte interaction. This study examines whether a leucocyte filter removes activated granulocytes or a general leucocyte population.

Eleven patients undergoing cardiopulmonary bypass surgery were included in this study. Leucocyte filtration was achieved before the reperfusion phase with a Pall non-woven polyester filter located at the venous side of the heart-lung machine. After filtration, the trapped granulocytes inside the filter were examined with light and scanning electron microscopy and immunologically by CD45RO antigen binding to the filter material. Furthermore, leucocyte release markers were measured to determine whether cells were activated during filtration.

On microscopic evaluation it was found that 84% granulocytes and 14% lymphocytes were trapped in the filter, compared with 78% granulocytes and 22% lymphocytes in the blood before filtration. Granulocytes were trapped significantly more in the first blood contact layer of the filter material than in the middle layer and last layer, whereas lymphocytes trapped slightly more in the middle layer. The near maximum level of CD45RO expression was measured on granulocytes trapped inside the filter material, whereas CD2 and CD19 measured on lymphocytes were bound to a minor extent. ß-Glucuronidase concentration did not increase after filtration, suggesting the absence of activation of granulocytes by filtration.

The results of this study suggest that a leucocyte filter made of non-woven polyester material removes the activated granulocytes rather than leucocytes at random. This implies that this particular type of leucocyte removal filter is suitable for use in cardiopulmonary bypass patients whose granulocytes in the circulation are activated. Furthermore, measurement of activated granulocytes instead of total leucocyte count is likely preferable for functional assessment of leucocyte removal devices.
INTRODUCTION

Foreign materials used in the Cardiopulmonary bypass (CPB) circuit during heart surgery activate leucocytes, resulting in increased cell adhesiveness, release of oxygen radicals and enzymes, and finally, damage to the host. Primarily the neutrophilic granulocyte fraction is activated after initial contact with extracorporeal surfaces. It has been suggested that removal of these activated granulocytes by filtration reduces morbidity after heart surgery. However, during leucocyte filtration in CPB procedures, we observed a large patient-related variation in filtration efficiency. Based on the suggested mechanism of leucocyte removal by synthetic filters – that is, by adhesion to the filter material rather than by sieving – we speculated that this variation in filtration efficiency was related to a difference in the expression of leucocyte receptors, leading to a difference in adhesion capacity to the filter material. However, it has never been reported, by directly studying the leucocyte-filter interaction, whether during CPB, leucocyte filters remove granulocytes at random or remove primarily an activated subset of granulocytes. Therefore, we designed this study to examine whether leucocyte filters remove a large portion of activated granulocytes or a general leucocyte population. To achieve this goal, we used a filter during 14 minutes in the clinical setting of CPB and performed electronic cell count and biochemical tests on blood samples taken before and after filtration. Additionally, we performed histologic examination on embedded filter material and immunologic tests to show the presence of ‘activation receptors’ on leucocytes trapped inside the filter and on whole blood samples before and after filtration.

METHODS

Patients

After approval was received from the ethical committee and informed consent was received from patients, 11 patients undergoing an elective heart operation for either coronary artery bypass grafting or heart valve replacement were included in the study. Exclusion criteria were a history of allergy or recurrent infection, reoperation, and emergency operation. The characteristics of the patients are summarized in table 1.

Heart operation procedure

Anaesthesia was induced and maintained by intravenous infusion of sufentanil citrate (1 to 3 μg/kg) and midazolam (0.05 to 0.1 mg/kg). Muscle relaxation was achieved with pancuronium bromide (0.1 mg/kg). Cefamandol at a dose of 2 g and dexamethasone at a dose of 1 mg/kg were administered after induction. Anticoagulation was achieved by intravenous administration of bovine lung heparin at a dose of 300 IU/kg approximately 5 minutes before the start of CPB. Anticoagulation was monitored by Celite activated clotting time (International Technidyne Co, Edison, NJ). After CPB, heparin was neutralized by protamine chloride (3 mg/kg). The heart-lung machine consisted of roller pumps (Stöckert Instrumente GMBH, Munich, Germany) and a microporous polypropylene membrane oxygenator (CML Excel; Cobe Laboratories Inc., Lakewood, CO). Within 10 minutes of CPB initiation at a flow rate at 2.4 L/min/m², the
Table 1. Characteristics of patients (n = 11) receiving filtration.

<table>
<thead>
<tr>
<th>Variable</th>
<th>unit</th>
<th>mean (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Y</td>
<td>63 (47 - 76)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Height</td>
<td>Cm</td>
<td>175.1 (165 - 195)</td>
</tr>
<tr>
<td>Weight</td>
<td>Kg</td>
<td>79.6 (60 - 103)</td>
</tr>
<tr>
<td>CPB time</td>
<td>min</td>
<td>87.7 (56 - 165)</td>
</tr>
<tr>
<td>X-clamp time</td>
<td>min</td>
<td>60.5 (38 - 103)</td>
</tr>
<tr>
<td>Lowest temperature (°C) during CPB</td>
<td>°C</td>
<td>30.2 (27.6 - 31.7)</td>
</tr>
<tr>
<td>CABG</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>AVR</td>
<td></td>
<td>2</td>
</tr>
</tbody>
</table>

CPB, cardiopulmonary bypass; X-clamp, aortic cross-clamp; CABG, coronary artery bypass grafting; AVR, aortic valve replacement.

The aorta was cross-clamped and 1 L St. Thomas cardioplegic solution (4°C) was infused into the aortic root to provide myocardial preservation. During CPB, moderate hypothermia was induced (table 1). The mean arterial pressure was maintained at 50 to 60 mmHg during CPB.

**Leucocyte filtration**

Leucocyte filtration was achieved by using a prototype leucocyte removal filter (B 1320A; Pall Biomedical, Portsmouth, England). This was a redesign of the prototype filter used for our previous study to make it easier for clinical handling. The filter was incorporated in the circulation, in a parallel circuit at the venous site of the heart-lung machine, and one of the roller pumps (Stöckert) was used to maintain a flow rate of 500 mL/min. Leucocyte filtration was performed during the rewarming phase at the end of CPB just before release of the aortic cross-clamp and lasted for approximately 14 minutes. During filtration the pressure at the inlet side of the filter averaged 74 ± 17.5 mmHg.

**Blood sampling**

Blood samples were taken before and after filtration from the radial artery of the patients and every 2 minutes during filtration from the inlet and outlet sides of the filter. The blood specimens were collected in sodium citrate (0.32%). Leucocyte counts were performed with an electronic cell counter (Cell-Dyn 610; Abbott, Santa Clara, CA) to assess leucocyte removal by the filter. The relative cell removal rate was calculated every 2 minutes according to the following formula: relative cell removal rate = (1-[post-filter count/pre-filter count]) x 100. The average cell removal was calculated as a mean of the relative removal rates. The total number of removed cells was calculated by multiplying the absolute number of removed cells per liter (post filter count minus pre-filter count) with the volume of filtered blood. For biochemical assays, plasma was obtained by centrifuging of whole blood at 4°C for 10 minutes at 1100g, whereafter plasma was stored at -80°C until further examination. β-glucuronidase, a release product of activated granulocytes, was determined by an enzymatic assay (photospectrometry; Boehringer,
Mannheim, Germany) in plasma samples from the inlet and outlet sides of the filter, after 8 minutes of filtration, to indicate activation of granulocytes by the filter material. Platelet activating factors inhibiting capacity (PAF-IC) was also determined from the inlet and outlet sides of the filter after 2 minutes filtration by turbidometry in an aggregometer (Chrono-Log, Havertown, PA) to indicate platelet activating factor (PAF) production by activated leucocytes. The measurement of PAF-IC was conducted with platelets isolated from citrated platelet-rich plasma containing indomethacin (50 μg/mL) from the blood of a healthy volunteer by filtration through Sepharose CL-2B (Pharmacia Biotech Inc., Stockholm, Sweden). These platelets were resuspended in saline to a final platelet concentration of 50 x 10⁹/L and added to the plasma of the study patients. The maximum velocity of platelet aggregation was measured after PAF C16 (Cayman Chemical, Ann Arbor, MI) addition and was used to indicate the PAF-IC of the patients’ samples. Because the PAF-IC is maximal in normal plasma and reduces after PAF formation, normal human plasma was used as a negative control and saline as a positive control, resulting in no aggregation of the platelets and maximum aggregation of the platelets, respectively.

**Morphologic examination of leucocyte entrapment**

Nine leucocyte filters were collected immediately after CPB and were prepared for histological examination to enable differential leucocyte counting in the cross-section of the filter material. After release of the residual blood, the filters were perfused in their indicated flow direction with 500 mL of normal saline solution under a constant pressure of 75 mmHg to wash away the unbound leucocytes. This low perfusion pressure did not exceed the clinical filtration pressure and was chosen to prevent the release of attached leucocytes by high shear forces. To further control the stability of leucocyte binding within the filter, part of the filter material was washed again after the standard procedure with 3 L of normal saline solution under similar perfusion pressure. In each filter, leucocytes were counted in 3 different layers of the cross-section. This comparison showed that washing the filter with 500 mL of normal saline solution did not differ significantly with results when washing with 3 L of saline solution in regard to bound leucocytes (table 2) and thus was used as a standard procedure for the future experiments. After washing, the filters were cut open without damaging the filter material, and within 60 minutes after filtration, partly fixated in 4% paraformaldehyde 0.1 mol/L phosphate buffer (pH 7.4) and stored at -20°C. In duplicate, samples of the fixed filter material were dehydrated with alcohol and distilled water and embedded in plastic (GMA, Technovit 8100). Series of three slices of 2 μm were cut out of the cross-section of both samples of the plasticized filter material with a microtome (Jung 1140) and a D-knife with a Tungsten Carbide edge (16/20). Thus in total, 6 samples of each filter were prepared for light microscopy. All slices were stained by standard histologic methods with May-Grunwald-Giemsa and viewed under the microscope. Leucocytes in each slice were counted on three locations of the cross-section of the filter material. As the first location, the first layer of the filter material touched by blood was chosen. As the second location, the middle layer of the cross-section was viewed. As the third location, a microscopic view of the last layer, which bordered on the outlet of blood from the filter, was examined. Differential counting for segmented neutrophilic granulocytes,
Table 2. Mean differential microscopic leucocyte count in three layers between two groups

<table>
<thead>
<tr>
<th></th>
<th>Normal Washed*</th>
<th>Extra Washed*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Segmented neutrophils</td>
<td>278.6</td>
<td>86.6</td>
</tr>
<tr>
<td>band neutrophils</td>
<td>16.4</td>
<td>7.7</td>
</tr>
<tr>
<td>basophilic granulocytes</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>eosinophilic granulocytes</td>
<td>17.6</td>
<td>2.0</td>
</tr>
<tr>
<td>lymphocytes</td>
<td>25.2</td>
<td>28.2</td>
</tr>
<tr>
<td>monocytes</td>
<td>9.6</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Samples were washed according to protocol with 500 mL saline solution (n = 24, 3 different filters), and samples were extra washed with 3 L saline solution (n = 18, 3 different filters). No statistically significant differences were observed between these two washing procedures.

*layers: 1, first blood contact layer; 2, middle layer; 3, last blood contact layer.

band neutrophilic granulocytes, lymphocytes, monocytes, eosinophilic and basophilic granulocytes was done in one microscopical field (enlargement x400) under the condition that at least 100 cells were counted in one layer. For scanning electron microscopy, pieces of filter material out of the same three layers that had been fixed were used. Postfixation was performed with 1% OsO₄ in phosphate-buffered saline solution for 3 hours, followed by dehydration in ethanol series. After critical point drying with CO₂, the samples were supercoated with gold and examined with scanning electron microscopy at 2 kV (Jeol 6301F, Tokyo, Japan).

Immunologic examination of activated granulocyte entrapment

The freezer-stored non-fixed parts of the filters were examined for the binding of specific antibodies to cells trapped inside the filter material. The antibodies used for this procedure were labeled with europium Eu-DDTA (Wallac, Turku, Finland), which allows sensitive detection by means of time-resolved fluorescence. Mouse anti-human CD45RO monoclonal antibody (Caltag Laboratories, San Francisco, CA) was used as a marker for the specific binding of activated granulocytes. To estimate the amount of CD45RO binding to T and B cells, specific antibodies against T-cell receptors (mouse anti-human CD2 monoclonal antibody; Caltag Laboratories) and against B-cell receptors (mouse anti-human CD19 monoclonal antibody; Caltag Laboratories) were used. To measure the binding of the antibody to the cells trapped inside the filter material, materials were separated in three layers representing the same specific locations of the cross-section of the filter material used in the morphologic examination. Each layer was divided into three parts. This resulted in nine parts per filter to be tested. Each part was carefully weighed to correct the amount of antibody binding for filter mass. Then, through a standard procedure, each part was washed with saline solution and incubated for 30 minutes with Eu-labeled antibody on a plateshaker and for 5 minutes with 3% H₂O₂. After the non-bound antibody was washed away, the Eu was released in enhancement fluid and counted in an Arcus (Wallac). A negative reference was made during each test by triplicate measurement of the nonspecific antibody binding to a sample of non-used filter material that had been incubated for 60 minutes in leucocyte-
and platelet-free plasma. A positive reference was made on filter material samples from 3 patients to test the maximum CD45RO binding to the filter material; Zymosan-activated plasma (Sigma, St. Louis, MO) containing high concentrations of C5a was incubated with the filter material for 20 minutes before CD45RO antibody binding.\textsuperscript{19,20}

In addition, removal of activated leucocytes from the blood of patients during CPB was tested by flow cytometric measurement of the adhesive receptor present on activated granulocytes (CD11b; DPC, Los Angeles, CA) in blood before and after the filter. Thus from 3 patients, blood samples were taken before filtration from the radial artery, before and after passing the filter at 2 and 10 minutes filtration from the afferent and efferent lines of the filter, and after the filtration procedure from the radial artery. Immediately after collection in sodium citrate (0.32%) blood was incubated with phyco-erythrin-labeled anti-CD11b, treated with Optilyse C (Immunotech, Marseilles, France), and prepared for flowcytometric analysis (FACS, Coulter, Luton, England).

Statistics

Before data analysis, all non-categorical data were tested and found normal distributed according to the Kolmogorov-Smirnov goodness-of-fit test. An unpaired two-tailed Student $t$ test was used to test the differences between the different leucocyte counts, non-categorical patient characteristics and immunologic data. An unpaired one tailed Student $t$ test was used to test the difference between the rinsed and extra-rinsed microscopical leucocyte counts. To detect possible differences between microscopic cell counts in the three different layers in cross-section, one way analysis of variance was used to compare groups. Duncan’s multiple comparison post hoc procedure was used to quantify any differences among groups that were found to be significant. A value of $p < 0.05$ was considered statistically significant. All haematologic, morphologic, immunologic and biochemical data are expressed as mean and standard error of the mean, unless otherwise indicated.

RESULTS

Morphologic examination of leucocyte entrapment

Under light microscopy, the number of segmented neutrophilic granulocytes, band neutrophils, monocytes and eosinophilic and basophilic granulocytes was significantly reduced along the flow direction through the three layers of the cross-section of the filter material (table 3). The middle and last blood contact layer showed statistically significantly fewer granulocytes than the first layer ($p < 0.0001$). Lymphocyte counts, however, did not differ significantly between the first two layers but were significantly reduced in the last layer (table 3). In total over 3 layers, granulocytes comprised 84\% of the total microscopically counted leucocytes. Lymphocytes attributed 14\% to the total counted leucocytes. The electronically measured composition of leucocytes in blood before filtration was 78\% granulocytes and 22\% lymphocytes. Massive adhesion of granulocytes to the filter material was also shown by scanning electron microscopical pictures of the filter material after use in the clinic (figure 1). Additional electronic leucocyte counting in blood revealed an average leucocyte removal during the first 10
Table 3. Different microscopic leucocyte counts in 3 layers of the cross-section of the filter material.

<table>
<thead>
<tr>
<th></th>
<th>count in cross-section*</th>
<th></th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>segmented neutrophils</td>
<td>294.5 ± 23.29</td>
<td>61.5 ± 21.92</td>
<td>26.2 ± 10.69</td>
<td>0.0001</td>
</tr>
<tr>
<td>band neutrophils</td>
<td>13.0 ± 1.88</td>
<td>3.3 ± 1.63</td>
<td>1.3 ± 0.81</td>
<td>0.0001</td>
</tr>
<tr>
<td>basophilic granulocytes</td>
<td>1.6 ± 0.33</td>
<td>0.3 ± 0.15</td>
<td>0.1 ± 0.07</td>
<td>0.0001</td>
</tr>
<tr>
<td>eosinophilic granulocytes</td>
<td>11.4 ± 2.27</td>
<td>1.0 ± 0.53</td>
<td>0.2 ± 0.12</td>
<td>0.0001</td>
</tr>
<tr>
<td>lymphocytes</td>
<td>22.4 ± 2.47</td>
<td>28.2 ± 2.25</td>
<td>17.5 ± 2.51</td>
<td>0.0158†</td>
</tr>
<tr>
<td>monocytes</td>
<td>8.5 ± 1.00</td>
<td>1.7 ± 0.63</td>
<td>0.7 ± 0.35</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Each layer was counted in total 102 times in 9 patients (n = 9). Counts are expressed as mean and standard error per layer. P-values indicate difference between the 3 layers after Duncan’s post hoc testing. Layers: 1, first blood contact layer; 2, middle layer; 3, last blood contact layer; †, significance was found only between the middle and last layer.

Figure 1. Electron microscopic pictures showing the filter material and trapped cells from a patient who received filtration (A magnification x290, B magnification x2900). Granule-rich neutrophils are clearly distinct from the red blood cells after the dehydration process in preparation for electron microscopy.
The minutes of filtration of 79% (table 4). Granulocyte removal was 84% and lymphocyte removal 73% (table 4). In five sequential measurements during the first 10 minutes a gradual decrease of leucocyte removal, from 91% to 70% (table 4), was observed. However, in spite of 10 minutes leucocyte filtration, the average systemic leucocyte counts measured in 11 patients did not change during the period of filtration (before filtration $3.68 \pm 0.42$, after filtration $3.62 \pm 0.49$; $p$-value 0.94).

**Immunologic examination of activated granulocyte entrapment**

A significant increase in CD45RO binding to the filter material after leucocyte filtration in comparison with the non-specific binding without leucocytes was found ($P<0.001$, figure 2). In the middle layer no increase was found in the CD45RO expression after further stimulation of granulocytes with Zymosan-activated plasma (figure 2). In the first and last layers, however, CD45RO expression increased after Zymosan stimulation (figure 2). Microscopic granulocyte count, CD45RO binding, and maximal CD45RO binding to the filter decreased significantly along the flow direction. Proving the validity of the test, the decrease of microscopic granulocyte counts correlated best with the decrease in maximal CD45RO expression. A significant CD2 binding (T cells) to the three different filter layers as compared with the non-specific binding was discovered. CD19 binding (B cells), however, was significantly different only from the non-specific binding in the middle layer. The average CD2 binding was, in accordance to the microscopic lymphocyte count, highest in the middle layer, although no significant difference was found between the layers (figure 3).

The additional antibody tests by means of flowcytometric assessment on whole blood samples showed a clearly detectable CD11b expression in blood before filtration, whereas after filtration the CD11b expression was below the detection limit. The

<table>
<thead>
<tr>
<th>time points (min)</th>
<th>unit</th>
<th>leucocytes</th>
<th>granulocytes</th>
<th>lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>%</td>
<td>90.6 ± 3.0</td>
<td>92.7 ± 3.4</td>
<td>89.9 ± 3.5</td>
</tr>
<tr>
<td>4</td>
<td>%</td>
<td>83.1 ± 5.5</td>
<td>87.1 ± 6.6</td>
<td>79.9 ± 4.2</td>
</tr>
<tr>
<td>6</td>
<td>%</td>
<td>78.0 ± 6.9</td>
<td>82.5 ± 8.0</td>
<td>72.6 ± 5.5</td>
</tr>
<tr>
<td>8</td>
<td>%</td>
<td>71.5 ± 8.5</td>
<td>78.3 ± 9.8</td>
<td>64.9 ± 6.3</td>
</tr>
<tr>
<td>10</td>
<td>%</td>
<td>69.7 ± 8.4</td>
<td>75.3 ± 9.3</td>
<td>56.9 ± 8.4</td>
</tr>
<tr>
<td>0 - 10</td>
<td>%</td>
<td>79.0 ± 4.0</td>
<td>83.5 ± 3.6</td>
<td>73.4 ± 6.6</td>
</tr>
<tr>
<td>0 - 10</td>
<td>$10^9$ / 5 L</td>
<td>12.6 (4.2 - 19.4)</td>
<td>9.8 (4.9 - 16.4)</td>
<td>3.0 (1.1 - 4.7)</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error, in percentage of the cell count measured before and after the filter, at 5 different time points during filtration ($n=11$). Additionally, the average removal of leucocytes during 10 minutes filtration in percentage (mean ± standard error) and the total absolute number ($10^9$ / 5 L blood) of removed leucocytes (number + range) in 10 minutes, are shown.
**Figure 2.** Granulocyte and CD45RO binding to the filter material in 3 different layers. I, first blood contact layer; II, middle layer; III, last blood contact layer. Specific CD45RO binding (calculated as CD45RO binding minus the non-specific binding) to the filter material is shown in black. Maximal CD45RO binding (after activation of the cells with zymosan-activated plasma) (white bars) was higher in the first and third layer than the specific binding without stimulation. The specific binding was significantly different from the non-specific binding (25,000 ± 6000, not shown). Granulocytes were counted in one microscopic field.

**Figure 3.** Lymphocyte and CD2 and CD19 binding to the filter material in 3 different layers. I, first blood contact layer; II, middle layer; III, last blood contact layer. Specific CD2 binding to the filter material (calculated as CD2 binding minus the non-specific binding: 57,000 ± 2000, not shown) was significantly increased as compared with the non-specific binding (*p < 0.01). CD19 binding (calculated as CD19 binding minus the non-specific binding: 73,000 ± 2000, not shown), was significantly increased in the middle layer (*p < 0.05). Lymphocytes were counted in one microscopic field.
removal of CD11b expressing cells, however, did not result in a significant reduction of the CD11b expression of leucocytes remaining in the systemic circulation after filtration. The background count, measured by a control antibody remained during the whole procedure less than 4% of the initial count before filtration.

Other biochemical measurements in blood samples

β-glucuronidase, measured in 8 patients, was 0.17 ± 0.02 before the filter and 0.17 ± 0.03 after the filter (p = 0.82), indicating no activation of granulocytes by the filter material or of granulocytes attached to the filter material. Furthermore, PAF-IC was not reduced after the filter as compared with before the filter.

DISCUSSION

The removal of leucocytes during CPB has been reported to reduce postoperative complications after heart surgery. It is not known, however, whether this reduction is due to the removal of activated granulocytes or to at-random removal of leucocytes. This study showed that the filter removed mainly the activated granulocytes, as indicated by the following findings. First, CD45RO, a specific marker for activated granulocytes showed a significant and almost maximum amount of binding to the filter material, indicating that the majority of granulocytes trapped inside the filter material was activated. Second, a nearly complete reduction of CD11b, another marker of activated granulocytes, after the filter suggests the removal of activated granulocytes. Third, the granulocyte portion of total leucocytes inside the filter material was 84%, as compared to 77% in blood before filtration. The lymphocyte portion inside the filter material was 14% compared with 23% in the blood before filtration. Therefore the filter material trapped primarily granulocytes rather than lymphocytes.

The enormous CD45RO binding to the filter material in the first layer, where 75% of the granulocytes were trapped, was followed by a maximum CD45RO expression level in the middle layer. However, CD45RO binding to the middle layer might have been caused by the enhanced portion of lymphocytes. The last layer, in contrast, functioned less efficiently in respect to the amount of bound cells and the activation level. However, the CD45RO counts per granulocyte in the last layer were approximately two times higher than those in the first layer. The amount of CD45RO binding in the last layer might also have been enhanced by a relative increase in the percentage of lymphocytes (38% in the last layer as compared with 6% in the first layer). Absolute or relative enhancement by lymphocytes in the middle and last layer may therefore have been the cause of the discrepancy between the microscopically counted granulocytes and the CD45RO binding. The filter was composed of one layer that we artificially divided into three layers. Eliminating the last blood contact layer may have reduced the loss of lymphocytes. In patients undergoing CPB, lymphocytes are preferably preserved to maintain the host defence mechanism. Clearly a less-solid structure of the filter material would reduce the potential danger of flow obstruction by filter resistance. Even in a filter with a large pore size such as the one we used (figure 1), the accumulation of leucocytes might activate other blood elements, resulting in a cascade reaction that could obstruct the filter and damage the blood. The release of PAF might play a crucial role in activating platelets as the origin of a large clotting reaction. PAF levels caused by
this filter, fortunately, were not enhanced after filtration. Also coagulation may be enhanced by Factor X binding to CD11b, which is clearly expressed on the granulocytes during CPB.\textsuperscript{24}

Although CD45RO was originally used for detecting 45% of T and a few B cells, it was lately discovered that the antibody also binds to activated granulocytes.\textsuperscript{25-29} Since the filter material was especially designed for binding granulocytes, the expectation was that CD45RO would primarily be a marker for activated granulocytes. Given the results of the CD2, CD19 and microscopic leucocyte count in the filter material, it can also be concluded that CD45RO primarily measured the binding of activated granulocytes. CD2 binding to the filter material was not extensive and did not significantly differ among the three layers of the filter material. CD19, reflecting B cell binding did not even bind to the first and last blood contact layer. CD45RO, on the contrary, bound massively in accordance with the amount of microscopically counted granulocytes to the first layer and decreased in average in the middle and last layers. Therefore it can be concluded that this massive CD45RO binding to the first layer was at most 10% caused by the binding of lymphocytes. Furthermore, CD45RO is thought to be suitable marker for activated granulocytes, although this is not widely accepted.

The microscopic leucocyte counts of the filter material were also useful as controls for the antibody tests. In support of the validity of the antibody tests, and the CD2 and CD19 binding tests, the microscopical leucocyte counting tests showed a slight increased average binding of lymphocytes to the middle layer of the filter material. In addition, at a maximum CD45RO expression level of the granulocytes, Eu counts correlated perfectly with the number of microscopically counted granulocytes.

The leucocyte removal rate from circulating blood declined from 90% to 70% at the end of filtration, although large individual differences in the absolute numbers of removed cells existed among the patients (4.2 to 19.4 x 10\textsuperscript{9} / 5 liters blood). Therefore it is less likely that the filter lacked capacity for leucocyte adhesion; otherwise all patients would have demonstrated a similar total amount of removed leucocytes. A more plausible explanation would be the internalization of adhesion receptors by the filter material after the first pass, resulting in a decreased adhesive capacity of leucocytes contacting the filter material for the second time. The failure of leucocytes to adhere to artificial surfaces when exposed to monoclonal antibodies against CD11b receptors has been described.\textsuperscript{30} Also, the internalization of CD11b receptors after exposure to various stimuli has been reported.\textsuperscript{31-33} Thus it might be more wise to enlarge the first contact layer of leucocyte filters instead of the thickness of leucocyte filter material.

Although a considerable amount of activated granulocytes have been trapped in the filter, the systemic granulocyte counts did not change during the period of filtration. This is most likely due to the fact that the systemic rewarming of blood started in parallel with leucocyte filtration, which means that a new population of granulocytes entered the blood stream massively from the third space, extravascularly, and from the bone marrow. Other evidence supporting leucocyte entry into the blood stream was the finding of level amounts of granulocytes expressing CD11b in the pre-filter blood samples during the whole period of filtration, in spite of a clear removal of CD11b expressing cells. Actually, this newly released population of granulocytes in the circulation counteracts the reduction effect made by leucocyte filtration. Indeed, the reduction of systemic leucocytes by leucocyte filtration is obvious when compared with results in a group of
control patients whose increase in systemic leucocytes during the rewarming phase was more severe than in filtration patients.

It seems that the adhesion of granulocytes to the filter material did not result in further granulocyte activation, since there was no enhanced ß-glucuronidase, and PAF from stored granules. It demonstrates that the cellular structure remains intact during the relatively mild flow conditions in our venous bypass circuit.

In conclusion, we have shown that the activated part of the granulocytes and not leucocytes at random are removed by the presented type of leucocyte filter. These leucocyte removal filters may appear suitable for use in heart operation patients, whose granulocytes in the circulation are activated. Individual differences in granulocyte activation level might therefore be a plausible explanation for the large differences in filtration efficiency between patients. Moreover, the measurement of activated granulocytes instead of total leucocyte count might be preferable for assessment of leucocyte filter devices in the future. Furthermore, this study shows that the filter likely does not need a high capacity, since the activated granulocytes either adhere immediately or do not adhere at all. Finally, removal of activated granulocytes during CPB did not alleviate the patient’s exposure to activated granulocytes, since the remaining granulocytes and those from the marginating pool replaced the removed cells immediately. Therefore, to reduce the post-perfusion syndrome, repeated leucocyte filtration should be considered to enhance the efficiency of filtration.

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REFERENCES

