Assessment of removal of human cytomegalovirus from blood components by leucocyte depletion filters using real-time quantitative PCR.

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Running head: leucocyte depletion and cytomegalovirus removal

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ABSTRACT

To assess removal of CMV by leucocyte depletion (LD) filters, we developed a spiking model of latent virus, using peripheral blood mononuclear cells (PBMC) infected by co-culture with CMV-infected human fibroblasts. Infected PBMC were purified by dual magnetic column selection, then spiked into whole blood units or buffy coat pools prior to LD by filtration. CMV load and fibroblast contamination were assessed using quantitative CMV DNA real-time PCR and quantitative RT-PCR of mRNA encoding the fibroblast-specific splice variant of prolyl-4-hydroxylase, respectively. After correcting for fibroblast-associated CMV, the mean CMV load was reduced in whole blood by LD from $7.42 \times 10^2$ to 1.13 copies/µl ($2.81 \log_{10}$ reduction), and from $3.8 \times 10^2$ to 4.77 copies/µl ($1.9 \log_{10}$ reduction) in platelets. These results suggest that LD by filtration reduces viral burden but does not completely remove CMV from blood components.

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(136 words)
INTRODUCTION

Primary cytomegalovirus (CMV) infection results in life-long carriage of latent virus, notably in CD14+ monocytes\(^1\). Re-activation of donor CMV following transfusion\(^2\) is avoided by selecting CMV-seronegative donors for vulnerable patients. Since universal leucocyte depletion (LD) has been widely implemented, it is debated whether LD alone could provide equivalent protection\(^3,4\). Eight studies have shown no transmissions following pre-storage LD\(^5,6\), but a recent study reports increased transmission from LD CMV seropositive components\(^7\).

Quantitative real-time PCR has the dynamic range to assess CMV removal by LD\(^8,9\), but due to low copy number, we and others\(^9\) have failed to detect viremia in sero-positive donors and even optimal CMV PCR would require virtually all leucocytes in an LD component \(< 10^6\)\(^10\). We found techniques reported to increase CMV copy number (pollen\(^11\), \(\gamma\)interferon, hydrocortisone, GM-CSF and allogeneic cells\(^2\)) to be insufficiently robust for assessment of LD. We have previously used infected T-lymphocytes and real-time PCR to assess HTLV-I removal by LD\(^12\). A CMV-infected T-cell line has also been described to measure CMV removal \(^13\), but T-cells are not a major physiological reservoir of CMV. We have therefore modified a previously described system\(^14\) to generate CMV-infected mononuclear cells, including CD14+ monocytes, for spiking into blood donations prior to LD.
MATeRIALS AND METHODS

With Ethics Committee approval and donor consent, 450 ml blood donations from 6 CMV seronegative donors were collected into citrate phosphate dextrose. PBMC were prepared from a 24 ml aliquot by density gradient separation (mean yield $1.52 \pm 0.27 \times 10^7$), and cultured for 12 hours with $2 \times 10^6$ human embryonic fibroblasts infected with human CMV strain AD169. PBMC were separated from fibroblasts by double selection using magnetic beads coated with anti-human fibroblast antibodies then anti-CD45. Leucocyte subset distribution in a representative sample of recovered PBMCs was analyzed by dual label flow cytometry using murine PE anti-CD45, plus FITC anti-CD3, or FITC CD19 or FITC anti-CD14.

Following overnight hold at $4^\circ C$, each whole blood unit was spiked with its own CMV-infected PBMC, and LD undertaken using Pall WBF3 filters (Pall Europe, Portsmouth, UK), which were then backwashed with 60 ml saline. A similar approach was used with pooled platelet concentrates (Pall AutoStop filters), except that PBMC were prepared from pools of 4 CMV seronegative buffy coats, with mean yield $4.52 \pm 2.42 \times 10^7$ cells. Leucocyte counting (sensitivity 1 leucocyte/µl) was performed by flow cytometry (LeucoCOUNT, Becton Dickinson, Franklin Lakes, NJ).

CMV quantification (sensitivity 1 copy/µl) was performed by amplifying nucleotides 2410-2481 of Immediate Early (IE) gene DNA using real-time TaqMan PCR (ABI Prism 7700, Applied Biosystems); forward primer 5’CAAGAACTCAGCCTCCCTCAAGAC3’, reverse primer 5’TGAGGCAAGTTCTGCAATGC3’; TaqMan probe 5’CCAATGGCTGCAGTCAGGCCATG3’. For fibroblast quantification, reverse-transcription real-time PCR was developed to amplify exon 12a of mRNA encoding a fibroblast-specific splice variant of prolyl-4-hydroxylase $^{15}$; forward primer
5´GAACCGCAGTTCTCTCTCTTAG3´, reverse primer
5´CTCTCAGGGTCTCGCTCGTCC3´, TaqMan probe
5´TGATGAGCGACCTTTTGACAGCGG3´.

The size and sequence of PCR amplicons were confirmed. CMV IE DNA and prolyl-4-hydroxylase mRNA assays were linear over 4_{10}logs. To create a standard curve for fibroblast quantification, mRNA was extracted from dilutions of fibroblasts from 1.5 \times 10^3 – 1.5 \times 10^{-1} /mL, and assayed for prolyl-4-hydroxylase. The contribution of CMV-infected fibroblasts to the overall CMV signal was calculated from a standard curve constructed by diluting CMV-infected fibroblasts from 8 \times 10^4 – 8 \times 10^{-1}/mL in 4 \times 10^6 CMV negative mononuclear cells, and assaying each dilution for CMV DNA. This demonstrated a mean CMV load of 3 copies/fibroblast. After fibroblast quantification, the calculated fibroblast-associated CMV signal was subtracted from the total CMV copy number to obtain the CMV load attributable to PBMC’s at each stage.
RESULTS AND DISCUSSION

The mononuclear cell distribution in a single representative spiking sample was CD3+ 63%, CD14+ 11%, CD19+ 26%. Purification achieved mean fibroblast removal from whole blood and buffy coat-derived PBMC of 96.3 +/- 5.4% and 77.21 +/- 20.2% respectively, with corresponding CMV recoveries of 2.67 +/- 1.87% and 13.9 +/- 10.9%. The CMV signal attributable to fibroblasts in the whole blood spike was <1% in 5 samples, and 16% in the sixth, the corresponding figures for the platelet spike being <11% in 5 samples, and 37.7% in the sixth (Table 1).

All LD units contained <5 x 10^6 leucocytes/unit. After correcting for CMV attributable to fibroblasts, whole blood LD reduced the CMV load from a mean of 742 to a mean of 1.13 CMV genome copies/µl, a mean reduction of 2.81 +/- 0.31 10 log (Table 2). For platelets, mean CMV levels pre-and post-LD were 380 and 4.77 copies/µl respectively, a mean reduction of 1.9 +/- 0.64 10 log. The mean post-filtration plasma signal from whole blood and platelets was 1.52 (range 0.05-3.72) and 6.19 (range 1.09-12.8) copies/µl respectively. Back-washing whole blood and platelet filters resulted in means of 75 and 27% CMV recovery respectively.

This system permits assessment of CMV removal by filtration using fresh PBMC, including the relevant CD14+ population. The 2.8 log reduction in CMV with whole blood LD is consistent with previous findings using either CMV-infected T cells[^13], or CMV antigenemic blood with 10-100 times lower CMV load than used here[^16]. It was not possible to correlate CMV removal with residual leucocyte counts, since these were below the detection limit in all units. Although there were 2 samples in which the CMV contribution from fibroblasts was unusually high, this had no impact on the efficacy of the LD process. The incomplete CMV removal in our study cannot be attributed to filter
overload, since the spiked leucocytes were derived from the donation. We have not assessed removal of granulocyte-associated-CMV, but these are a minor source of latent virus\textsuperscript{11,23}.

We and others have previously reported >4_{10} log monocyte reduction by whole blood LD\textsuperscript{17,18}. The cause of the discrepancy between monocyte and CMV removal is not clear, but with HTLV-infected T cells and similar filters, overall T cell reduction was also 1 log greater than HTLV Tax removal\textsuperscript{12}. It may be that monocytes activated by CMV infection are removed less well by filters than non-infected cells. Evidence for possible monocyte activation comes from recent observations reporting cytokine production by PBMC’s exposed to CMV for only 18 hours, a CD14-dependent phenomenon\textsuperscript{19}. The less efficient removal of CMV by platelet than by whole blood filtration was also found with HTLV\textsuperscript{12}, although one study of filtration and centrifugal apheresis LD achieved approximately 3_{10} log CMV removal\textsuperscript{11}. We noted greater plasma contamination associated with reduced viral recovery from filters with platelet LD, raising the possibility of CMV release from leucocyte cytoplasm\textsuperscript{20} during filtration. Free CMV is not amenable to removal by LD filters\textsuperscript{21}, so release into plasma could provide a mechanism for transmission, as has been seen after post-storage LD\textsuperscript{22}. However, transient plasma viremia is seen in <10% cases of primary CMV infection\textsuperscript{23}, yet transmission by plasma or derivatives is not reported, suggesting that free CMV in plasma may not be infectious. To generate an adequate CMV signal, it was necessary to super-infect components to levels 280-600 times greater than in CMV carriers\textsuperscript{25}. If levels of CMV reduction demonstrated here applied in CMV carriers, LD components would contain no more than 0.01-0.1 viral copies/\mu l. Whether this residual level is sufficient to reliably prevent CMV infection in immunosuppressed recipients remains unclear. We measured genome copies, not infectious virus, and our short co-culture of PBMC’s with CMV-infected fibroblasts in the absence of monocyte
differentiation signals would not result in PBMC expression of viral genes or infectious virus which could be titred (JH Sinclair, personal communication). The balance of evidence from clinical studies still suggests that acceptable ‘CMV-safety’ can be achieved by pre-storage LD. However, the incomplete CMV clearance by filtration suggests a need for greater understanding of the mechanisms and limitations of CMV removal from LD components.

Text words 1261
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References


17. Pennington J, Garner S, Sutherland J, Williamson LM. Residual subset population analysis in WBC-reduced blood components using real-time PCR quantification of specific mRNA. Transfusion. 2001;41:1591-1600.


### SPIKING SAMPLES FOR WHOLE BLOOD UNITS

<table>
<thead>
<tr>
<th>CMV copies</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Mean (SD) Log copy number</th>
<th>Mean copy number</th>
</tr>
</thead>
<tbody>
<tr>
<td>After co-culture</td>
<td>1.64 x 10^9</td>
<td>2.52 x 10^8</td>
<td>1.42 x 10^9</td>
<td>5.92 x 10^8</td>
<td>1.53 x 10^9</td>
<td>2.75 x 10^8</td>
<td>8.86 (0.38)</td>
<td>7.26 x 10^6</td>
</tr>
<tr>
<td>After purification, in PBMC for spiking</td>
<td>5.08 x 10^7</td>
<td>4.69 x 10^6</td>
<td>2.90 x 10^7</td>
<td>3.70 x 10^7</td>
<td>2.50 x 10^7</td>
<td>3.19 x 10^8</td>
<td>7.22 (0.50)</td>
<td>1.65 x 10^7</td>
</tr>
<tr>
<td>% CMV recovery</td>
<td>3.1</td>
<td>1.86</td>
<td>2.04</td>
<td>6.25</td>
<td>1.63</td>
<td>1.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CMV signal in final spike attributable to fibroblasts</td>
<td>3.3 x 10^5</td>
<td>7.5 x 10^5</td>
<td>9.9 x 10^3</td>
<td>8.1 x 10^3</td>
<td>8.1 x 10^2</td>
<td>1.2 x 10^3</td>
<td>4.38 (1.11)</td>
<td>2.4 x 10^4</td>
</tr>
<tr>
<td>% CMV from fibroblasts</td>
<td>0.65</td>
<td>16</td>
<td>0.03</td>
<td>0.02</td>
<td>0.003</td>
<td>0.37</td>
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### SPIKING SAMPLES FOR PLATELET UNITS

<table>
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<tr>
<th>CMV copies</th>
<th>A</th>
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<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Mean (SD) Log copy number</th>
<th>Mean copy number</th>
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<tr>
<td>After co-culture</td>
<td>1.8 x 10^8</td>
<td>5.1 x 10^8</td>
<td>1.5 x 10^9</td>
<td>1.8 x 10^9</td>
<td>4.5 x 10^8</td>
<td>1.2 x 10^9</td>
<td>8.85 (0.38)</td>
<td>7.15 x 10^5</td>
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<tr>
<td>After purification, in PBMC for spiking</td>
<td>6.38 x 10^6</td>
<td>5.62 x 10^7</td>
<td>6.33 x 10^7</td>
<td>5.86 x 10^8</td>
<td>8.78 x 10^7</td>
<td>1.51 x 10^8</td>
<td>7.87 (0.72)</td>
<td>7.49 x 10^7</td>
</tr>
<tr>
<td>% CMV recovery</td>
<td>3.54</td>
<td>11</td>
<td>4.22</td>
<td>32.6</td>
<td>19.5</td>
<td>12.6</td>
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</tr>
<tr>
<td>CMV signal in final spike attributable to fibroblasts</td>
<td>2.4 x 10^6</td>
<td>1.44 x 10^6</td>
<td>6.6 x 10^3</td>
<td>2.9 x 10^6</td>
<td>9.6 x 10^6</td>
<td>2.4 x 10^5</td>
<td>5.86 (1.13)</td>
<td>7.31 x 10^5</td>
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<tr>
<td>% CMV from fibroblasts</td>
<td>37.7</td>
<td>2.56</td>
<td>0.01</td>
<td>0.51</td>
<td>10.9</td>
<td>0.16</td>
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</tbody>
</table>

**Table 1.** CMV recovery in the mononuclear cell preparation used to spike whole blood units (n = 6) and platelet units (n=6). The proportion of CMV signal attributable to fibroblast contamination is also shown. A mean of 3 CMV copies/fibroblast is used in the calculation.
### Whole Blood

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Mean (SD) cell number</th>
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</thead>
<tbody>
<tr>
<td>Fibroblasts/µl</td>
<td>Pre-LD 2.60</td>
<td>1.30</td>
<td>4.37</td>
<td>2.63</td>
<td>9.47</td>
<td>2.83</td>
<td>3.87 (2.91)</td>
</tr>
<tr>
<td></td>
<td>Post-LD 0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.15</td>
<td>0.00</td>
<td>0.00</td>
<td>0.03</td>
</tr>
<tr>
<td>Corrected CMV copies/µl</td>
<td>Pre-LD 4060</td>
<td>188.00</td>
<td>420</td>
<td>2880</td>
<td>1760</td>
<td>103</td>
<td>2.87 (0.66)</td>
</tr>
<tr>
<td></td>
<td>Post-LD 3.96</td>
<td>0.51</td>
<td>1.07</td>
<td>3.77</td>
<td>0.85</td>
<td>0.30</td>
<td>0.053 (0.45)</td>
</tr>
<tr>
<td>Log₁₀ CMV reduction</td>
<td>3.00</td>
<td>2.56</td>
<td>2.59</td>
<td>2.88</td>
<td>3.31</td>
<td>2.53</td>
<td>Mean (SD) log reduction 2.81 (0.31)</td>
</tr>
</tbody>
</table>

### Platelets

<table>
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<tr>
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<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Mean (SD) cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts/µl</td>
<td>Pre-LD 8.2</td>
<td>4.4</td>
<td>3.3</td>
<td>4.2</td>
<td>8.1</td>
<td>6.1</td>
<td>5.72 (2.09)</td>
</tr>
<tr>
<td></td>
<td>Post-LD 0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Corrected CMV copies/µl</td>
<td>Pre-LD 88.4</td>
<td>631</td>
<td>824</td>
<td>1960</td>
<td>57.9</td>
<td>598</td>
<td>2.58 (0.59)</td>
</tr>
<tr>
<td></td>
<td>Post-LD 0.44</td>
<td>2.06</td>
<td>2.19</td>
<td>77.9</td>
<td>4.26</td>
<td>17.9</td>
<td>0.679 (0.79)</td>
</tr>
<tr>
<td>Log₁₀ CMV reduction</td>
<td>2.30</td>
<td>2.49</td>
<td>2.57</td>
<td>1.40</td>
<td>1.13</td>
<td>1.52</td>
<td>Mean (SD) log reduction 1.90 (0.64)</td>
</tr>
</tbody>
</table>

**Table 2.** Contaminating fibroblasts and CMV copies in whole blood and pooled platelet units before and after LD (n = 6). The number of CMV copies is corrected for fibroblast contamination at 3 CMV copies/fibroblast.
Assessment of removal of human cytomegalovirus from blood components by leucocyte depletion filters using real-time quantitative PCR

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