Brief report

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

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To assess removal of cytomegalovirus (CMV) by leukocyte depletion (LD) filters, we developed a spiking model of latent virus using peripheral blood mononuclear cells (PBMCs) infected by coculture with CMV-infected human fibroblasts. Infected PBMCs were purified by dual magnetic column selection and 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 reverse transcriptase–polymerase chain reaction (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 7.42×10^2 to 1.13 copies per microliter (2.81_{10}log reduction) and from 3.8×10^2 to 4.77 copies per microliter (1.9_{10}log reduction) in platelets. These results suggest that LD by filtration reduces viral burden but does not completely remove CMV from blood components. (Blood. 2004;103:1137-1139)

Introduction

Primary cytomegalovirus (CMV) infection results in life-long carriage of latent virus, notably in $CD14^+$ monocytes.[1] Reactivation of donor CMV following transfusion[2] is avoided by selecting CMV-seronegative donors for vulnerable patients. Because universal leukocyte 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 prestorage LD,[5,6] but a recent study reports increased transmission from LD CMV-seropositive components.[7]

Quantitative real-time polymerase chain reaction (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 seropositive donors, and even optimal CMV PCR would require virtually all leukocytes in an LD component (less than 10^6).[10] We found techniques reported to increase CMV copy number (pollen,[11] γ-interferon, hydrocortisone, granulocyte-macrophage colony-stimulating factor [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 human T-cell leukemia virus-I (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.

Study design

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

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

CMV quantification (sensitivity, 1 copy per microliter) was performed by amplifying nucleotides 2410 to 2481 of immediate early (IE) gene DNA using real-time TaqMan PCR (ABI Prism 7700; Applied Biosystems, Warrington, United Kingdom); forward primer 5′-CAAGAACTCAGCCTTCCCTAAGAC-3′, reverse primer 5′-TGAGGCAAGTTCTGCAATGC-3′; TaqMan probe 5′-CCAATGGCTGCAGTCAGGCCATG-3′. 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′-GAACCGCACTTCGACTTCTCTAG-3′, reverse primer 5′-CTCTCAGGTTYCGCTCGTCC-3′; TaqMan probe 5′-TGATGAGCGACCTTTTGACAGCGG-3′.

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

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Table 1. CMV recovery in the mononuclear cell preparation used to spike whole blood units (n = 6) and platelet units (n = 6)

CMV copies	A	B	C	D	E	F	Mean log copy no. (SD)	Mean copy no.
Spiking samples for whole blood units								
After coculture	1.64×10^9	2.52×10^8	1.42×10^9	5.92×10^8	1.53×10^9	2.75×10^8	8.86 (0.38)	7.26×10^8
After purification, in PBMCs for spiking	5.08×10^7	4.69×10^6	2.90×10^7	3.70×10^7	2.50×10^7	3.19×10^6	7.22 (0.50)	1.65×10^7
% CMV recovery*	3.1	1.86	2.04	6.25	1.63	1.16	—	—
CMV signal in final spike attributable to fibroblasts	3.3×10^5	7.5×10^5	9.9×10^3	8.1×10^3	8.1×10^2	1.2×10^4	4.38 (1.11)	2.4×10^4
% CMV from fibroblasts†	0.65	16	0.03	0.02	0.003	0.37	—	—
Spiking samples for platelet units								
After coculture	1.8×10^8	5.1×10^8	1.5×10^9	1.8×10^9	4.5×10^8	1.2×10^9	8.85 (0.38)	7.15×10^8
After purification, in PBMCs for spiking	6.38×10^6	5.62×10^7	6.33×10^7	5.86×10^8	8.78×10^7	1.51×10^8	7.87 (0.72)	7.49×10^7
% CMV recovery‡	3.54	11	4.22	32.6	19.5	12.6	—	—
CMV signal in final spike attributable to fibroblasts	2.4×10^6	1.44×10^6	6.6×10^3	2.9×10^6	9.6×10^6	2.4×10^5	5.86 (1.13)	7.31×10^5
% CMV from fibroblasts§	37.7	2.56	0.01	0.51	10.9	0.16	—	—

The proportion of CMV signal attributable to fibroblast contamination is also shown. A mean of 3 CMV copies per fibroblast is used in the calculation. — indicates not applicable.

Mean % (SD): *2.67 (1.87); †0.65 (1.09); ‡13.9 (10.9); §8.64 (14.8).

from dilutions of fibroblasts from 1.5×10^3 to 1.5×10^{-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 8×10^4 to 8×10^{-1}/mL in 4×10^6 CMV-negative mononuclear cells and assaying each dilution for CMV DNA. This demonstrated a mean CMV load of 3 copies per 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 PBMCs at each stage.

Results and discussion

The mononuclear cell distribution in a single representative spiking sample was CD3, 63%; CD14, 11%; and CD19, 26%. Purification achieved mean fibroblast removal from whole blood and buffy coat–derived PBMCs 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 less than 1% in 5 samples and 16% in the sixth, the corresponding figures for the platelet spike being less than 11% in 5 samples and 37.7% in the sixth (Table 1).

All LD units contained less than 5×10^6 leukocytes per 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 per microliter, a mean reduction of $2.81 \pm 0.31_{10}$log (Table 2). For platelets, mean CMV levels before and after LD were 380 and 4.77 copies per microliter, respectively, a mean reduction of $1.9 \pm 0.64_{10}$log. The mean postfiltration plasma signal from whole blood and platelets was 1.52 (range, 0.05-3.72) and 6.19 (range, 1.09-12.8) copies per microliter, respectively. Backwashing 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 PBMCs, including the relevant $CD14^+$ population. The

Table 2. Contaminating fibroblasts and CMV copies in whole blood and pooled platelet units before and after LD (n = 6)

	A	B	C	D	E	F	Mean (SD)	Mean copy no.
Whole blood								
Fibroblasts per microliter								
Before LD	2.60	1.30	4.37	2.63	9.47	2.83	3.87 (2.91)*	—
After LD	0.00	0.00	0.00	0.15	0.00	0.00	0.03*	—
Corrected CMV copies per microliter								
Before LD	4060	188	420	2880	1760	103	2.87 (0.66)†	742
After LD	3.96	0.51	1.07	3.77	0.85	0.30	0.053 (0.45)†	1.13
Log_{10} CMV reduction	3.00	2.56	2.59	2.88	3.31	2.53	2.81 (0.31)‡	—
Platelets								
Fibroblasts per microliter								
Before LD	8.2	4.4	3.3	4.2	8.1	6.1	5.72 (2.09)*	—
After LD	0.00	0.00	0.00	0.00	0.00	0.00	0.00*	—
Corrected CMV copies per microliter								
Before LD	88.4	631	824	1960	57.9	598	2.58 (0.59)†	380
After LD	0.44	2.06	2.19	77.9	4.26	17.9	0.679 (0.79)†	4.77
Log_{10} CMV reduction	2.30	2.49	2.57	1.40	1.13	1.52	1.90 (0.64)‡	—

The number of CMV copies is corrected for fibroblast contamination at 3 CMV copies per fibroblast. — indicates not applicable.

*Mean cell number (SD); †mean log copy number (SD); ‡mean log reduction (SD).

2.8_{10} 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 to 100 times lower CMV load than used here.[16] It was not possible to correlate CMV removal with residual leukocyte counts, because 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, because the spiked leukocytes were derived from the donation. We have not assessed removal of granulocyte-associated CMV, but these are a minor source of latent virus.[11,17]

We and others have previously reported more than 4_{10}log monocyte reduction by whole blood LD.[18,19] 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.[12] It may be that monocytes activated by CMV infection are removed less well by filters than noninfected cells. Evidence for possible monocyte activation comes from recent observations reporting cytokine production by PBMCs exposed to CMV for only 18 hours, a CD14-dependent phenomenon.[20] The less efficient removal of CMV by platelet than by whole blood filtration was also found with HTLV,[12] although one study of filtration and centrifugal apheresis LD achieved approximately 3_{10}log CMV removal.[11] We noted greater plasma contamination associated with reduced viral recovery from filters with platelet LD, raising the possibility of CMV release from leukocyte cytoplasm[21] during filtration. Free CMV is not amenable to removal by LD filters,[22] so release into plasma could provide a mechanism for transmission, as has been seen after poststorage LD.[23] However, transient plasma viremia is seen in less than 10% of cases of primary CMV infection,[24] 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 superinfect components to levels 280 to 600 times greater than in CMV carriers.[25] If levels of CMV reduction demonstrated here applied in CMV carriers, LD components would contain no more than 0.01 to 0.1 viral copies per microliter. 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 coculture of PBMCs with CMV-infected fibroblasts in the absence of monocyte differentiation signals would not result in PBMC expression of viral genes or infectious virus that could be titrated (J. H. Sinclair, personal communication, 2003). The balance of evidence from clinical studies still suggests that acceptable "CMV safety" can be achieved by prestorage 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.

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