

Comparison of the effects of filtration leucapheresis and discontinuous flow centrifugation leucapheresis on granulocyte microbicidal function

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SUMMARY In an investigation of the in vitro phagocytic and microbicidal function of granulocytes collected by filtration leucapheresis (FL) from 18 donors and by discontinuous flow centrifugation leucapheresis (DFC) from six donors, comparison was made with the function of granulocytes obtained from the same donors by venepuncture and density gradient centrifugation over Ficoll-Isopaque (FI).

No significant impairment of the phagocytosis or killing of *Candida guilliermondii* by either FL- or DFC- granulocytes was observed. Although the ability of FL-granulocytes to phagocytose and kill *Staphylococcus aureus* did not differ significantly from the function of control FI-granulocytes, DFC-granulocytes were significantly less active.

The three most widely used methods of granulocyte collection for transfusion are filtration leucapheresis (FL), continuous flow centrifugation (CFC) and discontinuous flow centrifugation (DFC). FL provides good yields of granulocytes but morphology and functional integrity have variously been reported as abnormal by some,¹⁻⁴ normal by others.^{5,6} These discrepancies may reflect differences in collection techniques; in particular the time for which the granulocytes are in contact with the filter^{2,5,7,8,9} and the degree of tapping of the filters during elution.^{4,8} CFC and DFC supply a lower yield of granulocytes but their morphology and function have generally been reported as normal.²⁻⁵ However, Beaud *et al*¹⁰ demonstrated some impairment of DFC-granulocyte function related to the occurrence of degranulation during centrifugation.

For granulocytes to be optimally effective clinically it is important that they should maintain their functional integrity. The apparent differences in functional capacity of granulocytes harvested in different laboratories prompted us to investigate and compare the function of granulocytes harvested by FL and DFC.

Material and methods

LEUCAPHERESIS DONORS

Peripheral blood was donated by 24 healthy adults under 50 yr of age. Oral dexamethasone (8 mg) was administered 16 h before the donation.

COLLECTION OF GRANULOCYTES

Ficoll-Isopaque separated (FI) granulocytes In order to study the effect of leucapheresis on granulocyte function, FI-granulocytes were used as a control. Before leucapheresis 10-20 ml heparinised blood was obtained from the donor, layered over Dextran-Isopaque and the red cells sedimented for 60 min at 37°C.¹¹ Granulocytes were then separated from the leucocyte-plasma by centrifugation over Ficoll-Isopaque, SG 1.077, at 400 g for 25 min.¹¹ After washing in Hanks' balanced salt solution (HBSS) the granulocytes were resuspended at 5×10^6 /ml.

FL-granulocytes were harvested using the Fenwal Leukopak system. Donors received 2000-2500 U heparin intravenously at the beginning of the procedure. Filters were primed with up to 1000 ml normal saline containing 1 U/ml heparin. Blood flow rate through the filters was 60-65 ml/min and elution was begun not later than 2 h after commencement. Red cells were returned to the donor by rinsing the

filters with 500 ml normal saline containing 3 U/ml heparin and the granulocytes then eluted into 4 × 600 ml blood bags with 2000 ml fluid comprising 1570 ml normal saline, 400 ml fresh frozen plasma (FFP) and 30 ml ACD-A. Granulocytes were concentrated by centrifugation (400 g, 15 min at 4°C), pooled and resuspended to 200 ml in FFP. An aliquot from this suspension was washed in HBSS and resuspended to 5 × 10⁶ granulocytes/ml for function studies.

DFC-granulocytes were harvested using a Haemonetics Model 30 cell separator with a 225 ml collection bowl. The separator was set up in a standard manner with 500 ml 6% hydroxyethyl starch (HES) + 30 ml sodium citrate anticoagulant concentrate, containing 17 g sodium citrate dihydrate and 800 mg citric acid monohydrate, replacing the usual ACD anticoagulant. When this HES was finished it was then replaced with ACD formula B. The initial flow rate was 60–70 ml/min, being reduced to 20 ml/min just prior to and during cell collection. Six to eight cycles were performed, yielding 70 ml buffy coat/cycle to give 420–560 ml granulocyte concentrate. An aliquot from this suspension was washed in HBSS and the granulocytes resuspended to 5 × 10⁶/ml for function studies.

GRANULOCYTE FUNCTION STUDIES

All function studies were carried out within 1–2 h of completing the collection procedure.

Phagocytosis of candida

The technique of Yamamura *et al*¹² was adapted for use with *Candida guilliermondii* whereby the uptake of ³H-uridine by non-ingested candida was used as a measure of phagocytosis. Triplicate assays (5 × 10⁵ granulocytes + 5 × 10⁶ *C guilliermondii* in 0.5 ml HBSS + 10% AB serum) were incubated for 30 min at 37°C and phagocytosis then terminated by the addition of 1 mg phenylbutazone.¹³ Duplicate 0.1 ml aliquots were removed and the non-ingested candida quantified by labelling with 5-³H-uridine (0.5 μCi, 5 Ci/mmol: 30 min, 37°C). Candida-associated radioactivity was harvested on to GF/C glass fibre filters for liquid scintillation counting.

Killing of candida was assessed by the release of ⁵¹chromium from labelled *C guilliermondii*.¹⁴ Triplicate assays (5 × 10⁵ granulocytes + 1.5 × 10⁶ ⁵¹Cr-*C guilliermondii* in 0.5 ml HBSS + 10% AB serum) were incubated for 60 min at 37°C. Granulocyte activity was terminated and the granulocytes disrupted by the addition of 2.4 mg sodium deoxycholate and 0.02 mg DNase in 0.2 ml; supernatants were taken for gamma counting. Results were expressed as:

$$\%Cr \text{ release} = \frac{X - S}{T - S} \times 100$$

where T = total radioactivity in 0.2 ml assay.

S = spontaneous release of chromium from candida in absence of granulocytes

X = granulocyte-induced release of chromium

The % release gave a measure of the % candida killed.¹⁴

Phagocytosis of bacteria

Granulocytes (5 × 10⁵) and methyl-³H-thymidine labelled *Staphylococcus aureus* (5 × 10⁶) in 0.5 ml HBSS + 10% AB serum were incubated for 20 min at 37°C.¹⁵ Phenylbutazone (1 mg) was added to inhibit further phagocytosis¹³ whilst non-ingested bacteria were removed with lysostaphin (5 μg). Granulocyte-associated radioactivity was then harvested on to GF/C glass fibre filters for liquid scintillation counting. When compared with the total counts-per-min (cpm) in the assay, granulocyte-associated cpm gave a measure of the degree of phagocytosis.

$$\% \text{ phagocytosis} = \frac{\text{granulocyte-associated cpm}}{\text{total cpm}}$$

Killing of bacteria Granulocytes (5 × 10⁵) and *Staph aureus* (5 × 10⁶) in 0.5 ml HBSS + 10% AB serum were incubated for 20 min at 37°C. The number of bacteria remaining viable was determined by colony counting. From comparison with a control containing *Staph aureus* alone the percentage of the total bacteria killed could be calculated. Then:

$$\% \text{ granulocyte-associated } \frac{\% \text{ phagocytosis}}{\% \text{ total bacteria killed}} = \frac{\% \text{ granulocyte-associated } \text{Staph aureus killed}}{\% \text{ total bacteria killed}} \times 100$$

STATISTICAL ANALYSIS

Data were analysed using Student's *t* test for matched pairs.

Results

During the period of the study the yields of white cells for the two methods were as follows:

Filtration leucapheresis (n=136): total WBC mean, 1.6 × 10¹⁰ (SD 0.55; range 0.5–3.3); mean % granulocytes, 94 (SD 4.4; range 71–100) Haemonetics model 30 (n = 88): total WBC mean, 1.2 × 10¹⁰ (SD 0.36; range 0.4–2.1); mean % granulocytes, 65(SD 15.5; range 23–93).

GRANULOCYTE CANDIDACIDAL FUNCTION

No significant difference was observed in ability to phagocytose and kill *C guilliermondii* when either FL- or DFC-granulocytes were compared with pre-leucapheresis FI-granulocytes from the same donor. The results are shown in Table 1. For granulocytes

Table 1 *Candidacidal function of granulocytes harvested by filtration (FL) and discontinuous flow centrifugation (DFC) leucapheresis*

	Mean candida ingested (%)			Mean candida killed (%)		
	n	FI	L	n	FI	L
FL	6	67	66	18	56	55
DFC	5	77	73	5	68	63

n = number of donors.

FI = Ficoll-Isopaque separated granulocytes prior to donation.

L = granulocytes harvested by leucapheresis (FL or DFC).

Statistical analysis by Student's *t* test for matched pairs.

Table 2 *Bactericidal function of granulocytes harvested by filtration leucapheresis (FL)*

Donor	Staph aureus ingested (%)		Granulocyte-associated Staph aureus killed (%)	
	FI	FL	FI	FL
10	65	61	97	93
11	56	67	64	49
12	41	56	88	63
13	55	63	87	56
14	52	54	50	56
15	41	40	88	82
16	61	65	NT	NT
18	60	30	75	87
	Mean 54	55	78	69

FI = Ficoll-Isopaque separated granulocytes prior to donation.

FL = granulocytes harvested by filtration leucapheresis.

NT = not tested.

Statistical analysis by Student's *t* test for matched pairs.

Table 3 *Bactericidal function of granulocytes harvested by discontinuous flow centrifugation leucapheresis (DFC)*

Donor	Staph aureus ingested (%)		Granulocyte-associated Staph aureus killed (%)	
	FI	DFC	FI	DFC
20	46	24	83	93
21	55	49	75	51
22	51	42	88	71
23	69	48	71	54
24	63	45	NT	NT
	Mean 57	42	79	65

FI = Ficoll-Isopaque separated granulocytes prior to donation.

FL = granulocytes harvested using Haemonetics Model 30.

NT = not tested.

Statistical analysis by Student's *t* test for matched pairs.

harvested by filtration leucapheresis: mean % candida ingested, FL = 67% FI = 66%; mean % candida killed, FL = 56% FI = 55%. For granulocytes harvested using the Haemonetics Model 30: mean % candida ingested, DFC = 77% FI = 73%; mean % candida killed, DFC = 68% FI = 63%.

GRANULOCYTE BACTERICIDAL FUNCTION

For granulocytes harvested by filtration leucapheresis the ability to ingest *Staph aureus* did not differ significantly from that of corresponding control FI-granulocytes: mean % *Staph aureus* ingested, FL = 55% FI = 54% (Table 2).

The percentage of granulocyte-associated *Staph aureus* killed was less for FL-granulocytes (69%) than for control FI-granulocytes (78%) but this difference was not significant at the 5% level (Table 2).

For granulocytes harvested using the Haemone-tics Model 30 the ability both to ingest and kill *Staph aureus* was significantly reduced when compared with control FI-granulocytes: mean % *Staph aureus* ingested: DFC = 42% FI = 57%; mean % granulocyte-associated *Staph aureus* killed, DFC = 65% FI = 79% (Table 3).

Discussion

In this study the *in vitro* candidacidal function of granulocytes harvested by both FL and by DFC was normal. FL-granulocytes also showed a normal ability to ingest and kill *Staph aureus*. In contrast DFC-granulocytes showed a significantly reduced capacity to phagocytose and kill *Staph aureus* when compared with pre-leucapheresis granulocytes.

Although the function of granulocytes collected by continuous or discontinuous-flow centrifugation has generally been found normal,²⁻⁵ there has been a report of reduced morphological and functional integrity in DFC-granulocytes.¹⁰ The results of this present study provide further evidence that the function of granulocytes harvested using DFC may be functionally impaired, possibly as a result of degranulation induced during centrifugation.¹⁰ However, it should be stressed that while the bactericidal function of DFC-granulocytes was significantly less than that of FI-granulocytes, all results were within our accepted normal range.

Granulocytes collected by FL have generally been reported as showing morphological and functional abnormalities¹⁻⁴ although some studies have found the cells to be normal.^{5,6} We have also found the function of FL-granulocytes to be normal. The discrepancies in the findings from different laboratories emphasise the importance of establishing a standard technique of collection. The reasons behind these differences may lie in the type of perfusion pump used, the rate of filter perfusion, the volume of blood perfused, the collection time, the type of eluting solution, the mechanism of elution and the solution in which the granulocytes are finally resuspended. There is evidence to suggest that the shorter the time of contact between granulocytes and nylon fibres, the greater the integrity of the harvested cells.^{2,5,7,8,9} It has also been shown that vigorous manual tapping during elution of the filters has a markedly adverse effect on granulocyte function.^{4,8} When granulocytes are eluted with vigorous tapping this can cause H₂O₂ release from resting cells, probably as a result of perturbation of the plasma mem-

brane.¹⁶ It is difficult to standardise the extent to which filters are tapped and this could explain much of the variation in results between laboratories and even between technicians within one laboratory. It is our practice to tap the filters gently during the elution procedure but Stegmann *et al*⁸ have proposed the development of a mechanical gentle-tapping system to minimise damage to granulocytes. Another important consideration is the final centrifugation to concentrate the granulocytes for transfusion. Centrifugation speeds have varied from 80 g to 2800 g.^{3,5,7,16} Centrifugation at a low g-force minimises damage to granulocytes. In order to optimise the harvesting conditions for FL-granulocytes we would suggest a collection time of less than two hours, gentle tapping of the filters used during elution with buffer at pH 6.5, concentration of granulocytes at 400 g and resuspension in FFP.

Although the clinical gain from granulocyte transfusions is difficult to evaluate it has been shown that filter-collected granulocytes can function normally in the recipient^{6,17} and Aisner *et al*¹⁷ have shown the clinical efficacy of FL-granulocytes to be superior to that of DFC-granulocytes.

There is evidence that granulocyte transfusions can favourably influence the outcome of life-threatening infections in patients with severe neutropenia;^{18,19} the value of prophylactic transfusions remains controversial.^{20,21} It is apparent that because techniques for harvesting granulocytes differ, and, therefore, the functional integrity of these cells may vary from laboratory to laboratory, comparison as to clinical efficacy should be made with caution.

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