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ARTIGOS

FLOW CYTOMETRIC QUANTITATION OF PHAGOCYTOSIS IN HEPARINIZED COMPLETE BLOOD WITH LATEX PARTICLES AND *CANDIDA ALBICANS*

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We report a rapid method for the flow cytometric quantitation of phagocytosis in heparinized complete peripherial blood (HCPB), using commercially available phycoerythrin-conjugated latex particles of 1µm diameter. The method is faster and shows greater reproducibility than Bjerknes' (1984) standard technique using propidium iodide-stained Candida albicans, *conventionally applied to the leukocytic layer of peripherial blood but here modified for HCPB. We also report a modification of Bjerknes' Intracellular Killing Test to allow its application to HCPB.*

Key-words: Phagocytosis. Flow cytometry. Latex particles. Candida albicans.

The phagocytic capacity of granulocytes 11 was until recently quantitated by extracting the leukocytic layer of peripheric blood, incubating it with *Candida albicans* and estimating phagocytosis through staining and examination under the light microscope[13]. The introduction of flow cytometry led to the development of more objective methods: in 1984, Bjerknes[5] described such a method using *Candida albicans*, with similar sensitivity to that obtained using the classical method. Bjerknes' test has since been applied in various experimental contexts[6 8 16 17]. In related studies[2 10], *Staphylococcus aureus* has been used for the measurement of both phagocytosis and hydrogen peroxide production, with similar results.

Bjerknes' test is conventionally applied to the leukocytic layer of peripherial blood. Here, we used heparinized complete peripherial blood (HCPB), to preveny the need for a cell separation step. We compared flow cytometric quantitation of phagocytosis using: a) propidium iodide-stained *Candida albicans* and b) commercially available phycoerythrin-conjugated 1µm diameter latex particles. Both tests were modified for a one-hour incubation period.

We are currently applying our modification of Bjerknes' test in routine analysis of the phagocytic capacity of polymorphonuclear leucocyte (PMNs) in peripherial blood of patients with immune disturbances.

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MATERIAL AND METHODS

Human leukocytes. We used 125µl of heparinized complete human peripheral blood (from 3ml of blood containing 0.05ml of heparin lithium salt at 10IU/ml), less than 3h postextraction, maintained at 20ºC, with a granulocyte count between 3,000 and 5,000 per mm^3.

Candida albicans. A sample of human origin was cultured in Sabouraud-chloramphenicol agar (Bio-Merieux®), and a subsample was harvested in PBS, pH 7.4, washed twice for 5 min at 500xg (Beckman J-6B). Viability was determined by trypan blue vital staining in a Neubauer chamber, and by flow cytometry with propidium iodide (PI) staining. Only batches with greater viability than 95% were used.

Opsonization was carried out, when required, by incubating 5ml of human pooled serum with 1ml of *Candida* suspension (250 x 10^7 cells/ml) for 45 min at 37ºC.

Fixation was carried out by mixing 70% ethanol with the *Candida* suspension (45:5 v/v) and incubating overnight at -20ºC. This fixation method was compared with three others, using an alkalin cation fixative, Triton 0.1% and Triton 0.5%. Viability was determined by PI staining and Epics Profile II (Coulter®) cytometre.

Propidium iodide labelling was carried out by incubating 100µl of the *Candida* suspension (both opsonized and non-opsonized) for 30 min with one of a series of mixtures containing 20, 40, 80, 160, 240, 400 or 600µg/ml of PI (in volumes of between 50 and 400µl) and 100µl of RNAse (1mg/ml). Flow cytometry showed

the 400µg/ml concentration to be most effective (data not show). Aliquots of the suspensions containing *Candida* labelled with PI at this concentration were stored at -20°C until use.

Candida *phagocytosis test (CPT)*. A mixture containing 125µl of HCPB and 25µl of the *Candida* suspension (containing 1 x 10^7 PI-labelled cells/ml, fixed with 70% ethanol as described above) was incubated at 37°C with continuous mechanical stirring for 30, 60, 90 and 120 min, and then fixed using the Epics Q-Prep leukocyte preparation system (Coulter®) prior to flow cytometry. Parallel experiments, all with 60 min incubation, were run with 15, 10 and 5µl of the *Candida* suspension. Phagocytosis was measured by flow cytometry (Coulter Profile II) of 1ml samples of the Q-Prep-fixed HCPB/PI-labelled *Candida* mixtures, with recording of red fluorescence.

Intracellular killing test (IKT). To determine optimum incubation time for the IKT, twenty-five-µl samples of unfixed *Candida* (1 x 10^7 cells/ml, viability by trypan blue vital staining > 95%) were incubated with 125µl of HCPB at 37°C with mechanical shaking for 30, 60, 90 or 120 min. Granulocytes were then fixed, and erythrocytes lysed, using the Q-Prep system, and the preparation was washed at 500xg with phosphate-bulfered saline (PBS), pH 7.4. The pellet was incubated for 5 min with 1ml of lysis buffer (2.5% sodium desoxycholate, pH 8.7, in sterile distilled water) to lyse granulocytes, then washed twice at 10,000xg with PBS. The pellet was resuspended in 1ml of PBS then incubated with 50µl of PI (400µg/ml) and 100µl of RNAse (1mg/ml) for 30 min at room temperature in a darkroom, to allow differentiation of dead and live cells. Analysis was by flow cytometry with measurement of red fluorescence.

Latex phagocytosis test (LPT). One-hundred-µl aliquots of 1µm diameter phycoerythrin (PE)-labelled latex particles (Polyscience®) 7 were washed twice with washing buffer (0.054 M glycine/saline, pH 8.2) at 13,000xg for 10 min (Beckman Microfuge 11). The pellet was resuspended in 5ml of washing buffer and sonicated for 30 sec with three 50W pulses (Branson Sonifier 250). The concentration of latex particles in this suspension according to the manufacturer's instruction was 9 x 10^8 particles per ml. Opsonization was achieved by adding 200µl of ammonium sulphate-purified immunoglobulins at 25mg/ml to 5ml of the suspension of sonicated latex particles, then incubating at 20°C with mechanical stirring for one hour. Following 2 - 3 washes in a washing buffer, the resulting pellet was resuspended in 5ml of storage buffer (0.27 M glycine/saline and 0.1% bovine serum albumin - BSA) and stored at 4°C. Phagocytosis of both opsonized and non-opsonized particles was quantitated by incubation of 5, 10, 20, 30 and 40µl of this suspension (containing 45 x 10^5, 9 x 10^6, 18 x 10^6, 27 x 10^6 and 36 x 10^6 latex particles respectively) with 125µl of HCPB at 37°C for 60 min, followed by fixation using the Q-Prep system. Flow cytometry measurement (FCM) was of orange fluorescence.

RESULTS

Candida *phagocytosis test*. The fixation method leading to the successful fixation of most *Candida* cells was with 70% ethanol at -20°C overnight (Table 1); 0.1% Triton and 0.5% Triton fixation methods were less effective. With a granulocyte-to-*Candida* cell ratio of 1:5, cell suspensions which had been labelled with a PI solution of 400µg/ml were found to give the most homogenous fluorescence peak (data no shows). The variation in phagocytosis percentages with different incubation times (Table 2) are in accordance with Bjerknes' results with the leukocytic layer of non-heparinized peripheric blood[5]. The optimum granulocyte-to-*Candida* cell ratio was 1:5 (i.e. 25µl of *Candida* suspension to 125µl of HCPB), with higher ratios leading to reduced phagocytosis (Table 3).

Latex phagocytosis test. Different volumes of a suspension containing 9 x 10^7 latex

Table 1 - Percentage of Candida *killed by the various fixatin methods, as determined by FCM (p < 0.01).*

	Control	Ethanol[a]	Alkaline cation[b]	Triton 0.1%[c]	Triton 0.5%[d]
Percentage %[e]	9.4 ± 0.1	90.5 ± 0.1	14 ± 0.2	12.8 ± 0.1	13.3±0.1

[a] incubation overnight with 70% ethanol at -20°C; [b] incubation for 15 min with 0.1M sodium acetate in a mixture of 10 mM Tris HCl pH 8.0 and 1mM EDTA at 30°C; [c] incubation for 15 min with 0.1% Triton X-100 (Sigma®) in PBS at 30°C; [d] incubation for 15 min with 0.5% Triton X-100 in PBS at 30°C; [e] mean ± SD; n = 3.

Table 2 - Percentage phagocytosis of propidium iodide labelled non-opsonized Candida, *as measured by FCM, following periods of incubation with HCPB.*

	Incubation period (min)[a]			
	30	60	90	120
Percentage phagocytosis[b]	58.8 ± 6.2	71.7 ± 11.2	82.3 ± 8.9	89.5 ± 6.7

[a] granulocyte/*Candida* cell ratio was 1:5.\; [b] mean ± SD; n = 10.

*Table 3 - Granulocyte-to-*Candida *ratios and percentage of phagocytosis obtained with 125µl of HCPB and different volumes of suspension of 70%-ethanol-fixed and PI-labeled* Candida *cells. Granulocyte count in the HCPB was berween 3,000 and 5,000 per mm3.*

volume added	*Candida* (1X10^8 cells/ml)				
	50µl	25µl	15µl	10µl	5µl
Grabulocyte: *Candida* ratio	1:10	1:5	1:3	1:2	1:1
% Phagocytosis[a]	40 ± 2.3	73 ± 4.3	45 ± 2.7	30 ± 3.2	9 ± 3.5

[a] mean ± SD; n = 5. ($p < 0.01$).

particles/ml were added to HCPB, with subsequent measurement of phagocytosis (Figure 1). The optimum granulocyte-to-latex particle ratio was 1:32 (20µl of the suspension containing 18 x 10^6 particles to 125µl of HCPB. The phagocytosis was much higher in HCPB (3ml of blood containing 0.05ml of the lithium salt of heparin at 10IU/ml) than in ethylenediaminetetraacetate, tripotassium salt (EDTA)-treated complete peripheric blood (3ml of blood containing 0.06ml of EDTA (K3) at 85g/l), in opsonized (OPS) and non-opsonized particles after 60 min incubation at 37°C (Table 4).

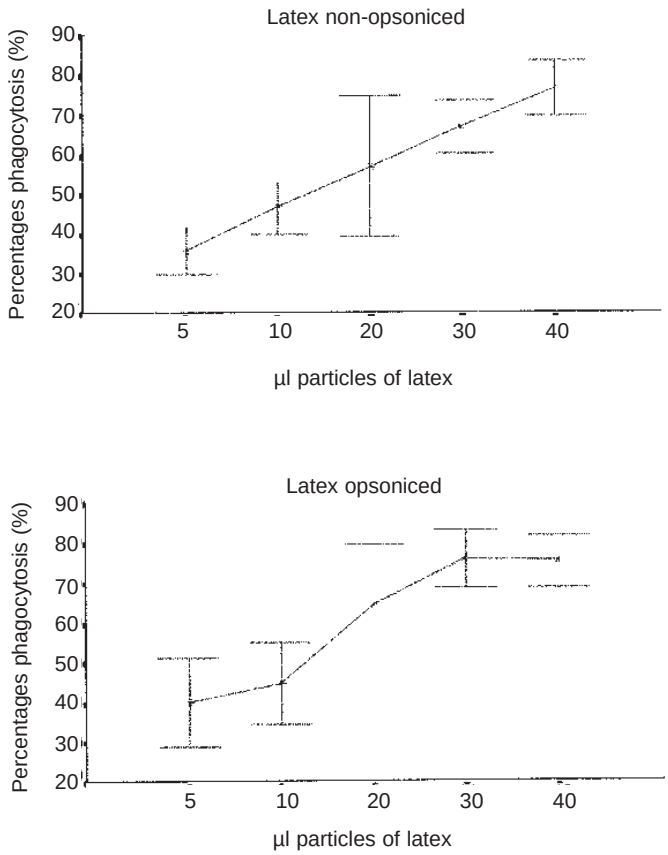

Figure 1 - Latex phagocytosis test: different volumes of a suspension containing 9 X 10^8 latex particles/ml were added to HCPB, incubated 60 min, with subsequent measurement of phagocytosis (mean ± SD; n = 20).

Table 4 - Percentage phagocytosis of opsonized and non-opsonized latex particles, as measured by FCM, following with heparinized blood (0.05ml sodium heparin at 10UI/ml) and EDTA (K) blood (0.06ml as 85g/l) in a total blood volume of 3ml with a 60 min period of incubation.

Percentage %[a]	Latex	
	non-opsonized	opsonized
EDTA (K3) blood	11.3 ± 0.2	15.3 ± 0.2
Heparinized blood	64.5 ± 0.2	76.2 ± 0.2

[a] mean ± SD; n = 3.

DISCUSSION

We have adapted Bjerknes' flow cytometric method for the quantitation of phagocytosis, which uses *Candida albicans* and the leukocytic layer of peripheric blood[4 5], for use with HCPB. This obviates the need for prior separation, washing and quantitation of the leukocytic layer, and is thus simpler and more rapid than Bjerknes' original method.

We have also developed an alternative test for the quantitation of phagocytosis in HCPB using commercially available PE-labelled latex particles, of smaller size and more homogenous morphology than *Candida* cells. Our results show that this test is considerably simpler, faster and more reliable than either Bjerknes' original method or our adaptation of it to HCPB, and we thus propose it as a valid alternative for routine quantitation of the phagocytic capacity of granulocytes in peripheric blood. Clearly, more complex methods are still necessary for detailed studies of patients with phagocytic disturbances[1].

Optimum granulocyte-particle ratio was found to be 1:5 in the case of the *Candida* test, and 1:36 in the case of the latex test.

The *Candida* test was additionally optimized with respect to incubation time, with the aim of ensuring sufficient phagocytosis to provide a strong fluorescent signal which is clearly differentiated from the background noise due to other forms of granulocyte-particle binding. In other respects, it was found that any departure from the standard incubation conditions (37ºC with continuous mechanical stirring) led to considerable increases in background noise.

We also attempted to determine the best PI concentration for *Candida* labelling such that the fluorescence signal was as strong and homogenous as possible. As has been noted in previous studies[17], homogeneity of staining is very difficult to achieve. Different batches of *Candida* cells fixed and stained under identical conditions respond unpredictably to storage, making it necessary to run controls every time the test is required. Likewise, the dispersion of both forward angle light scatter (FALS) and 90-degree light scater (90LS) diagrams were considerable even after filtration and homogenization with low-power ultrasound, giving high variability in granulocyte size once phagocytosis had occurred. All these problems are minimized when PE-labelled latex particles, which stain much more homogenously and have a much more constant size distribution (and consequently scatter diagrams which are easier to interpret)[12], are used.

Given the possibility that free (non-phagocytosed) labelled particles may cause fluorescence, leading to false positive results, we also ran controls in which suspensions containing labelled *Candida* cells (2.5 x 10^6 cells/ml) or latex particles (18 x 10^6 particles/ml) were treated and subjected to flow cytometry, as in the respective phagocytosis tests (CPT or LPT) but without incubation with blood. Counts of less than 1% of those obtained in the respective phagocytosis tests were recorded over periods of more than 120s (note that the standard phagocytosis test samples, containing about 15,000 PMNs, require between 90 and 120s to pass through the cytometer).

Our results confirm that blood treated with EDTA (K3) is not useful for phagocytosis quantitation because of the resulting complexation of calcium and consequent inhibition of calcium-dependent phagocytosis[14 15]. Our data likewise confirm the absence of phagocytosis by lympocytes.

The intracellular killing test depends upon the maintenance of a live *Candida* population in order to obtain a suspension of cells with viability greater than 90%. Propidium iodide will only stain those *Candida* cells with an altered cell wall which permits its diffusion. The two-stage lysis eliminates the problems associated with staining of cell debris, and the false positives which arise as a result.

We propose the use of the latex phagocytosis test for routine screening purposes, since it shows greater reproducibility than the *Candida* Phagocytosis Test and is less affected by differences in particle size and labelling/ storage conditions. Continued use of *Candida* for the intracellular killing test is, however, necessary. Under the test conditions described here, we observed no significant differences between phagocytosis of opsonized and non-opsonized latex particles; however, the application of both forms of the LPT during routine screening may be of value in showing up deficiencies in the opsonization capacity of the patient's serum.

RESUMEN

Se da cuenta de un método rápido para la cuantización del flujo citométrico de la fagocitosis en sangre periférica completamente heparinizada (HCPB), mediante la utilización de partículas de látex phycoerythrin-conjugadas de 1µm de diámetro disponibles comercialmente. El método es más rápido y presenta mayor reproducibilidad que la técnica estandar de Bjerknes' (1984) utilizando propidium iodide-teñida Candida albicans, *aplicada convencionalmente a la capa leucocitica de sangre periférica pero modificada por HCPB. Tambien damos cuenta de una modificación de Bjerknes' Intracellular Killing Test para permitir su aplicación a HCPB.*

Palavras-chaves: Fagocitosis. Flujo citométrico. Partículas de látex. Candida albicans.

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