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Phagocytic efficacy of macrophage-like cells as a function of cell cycle and Fcγ receptors (FcγR) and complement receptor (CR)3 expression

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Summary

Previous studies have shown that the efficiency of phagocytosis is a function of cell cycle and that phagocytosis promotes cell cycle progression. Because phagocytosis is dependent on cellular receptors we hypothesized that Fcγ receptors (FcγR) and complement receptors (CR) expression varied with cell cycle. Consequently, we used centrifugal elutriation of macrophage-like cells, fluorescence activated cell sorting analysis and receptor staining to investigate expression of FcγR and CR as a function of cell cycle. We confirmed that FcγR expression on macrophage-like cells increased as the cells progressed from G1 to G2 phases. Moreover, CR3 expression varied as a function of cell cycle in a manner similar to FcγR. Correlation of receptor expression with cell size showed that FcγR and CR3 expression on macrophages was determined largely by cell size enlargement during the cell cycle. The efficacy of both Fc- and complement-mediated phagocytosis of live *Cryptococcus neoformans* (Cn) showed a biphasic pattern with the efficacy of phagocytosis decreasing when the cells approached the G1–S interface, which paralleled the changes in receptor surface expression when cells exited G1 phase. Live Cn cells were significantly more resistant to phagocytosis than dead cells at all stages of macrophage-like cell cycle. In contrast to live cells, the efficacy of phagocytosis of dead Cn decreased as surface receptor expression increased. Hence, the efficacy of phagocytosis in this system as function of cell cycle is not related to phagocytic receptor expression.

Keywords: cell cycle, CR3, FcγR, macrophage, phagocytosis

Introduction

Eukaryotic cell cycle is divided into G1, S and G2 phases. Many genes, including those encoding DNA-synthesizing machinery proteins, growth factors and receptors, proto-oncogenes and some structural proteins, are expressed in a cell cycle-dependent manner [1]. Some of these genes play direct roles in cell replication while others are expressed at different times in the cell cycle. Among the latter, glucocorticoid receptor is functionally inactive in G2-M phase fibroblasts [2,3]. The expression of cell cycle-related genes can affect immunological function. In this regard, MHC II expression is not increased by interferon (IFN)-γ for macrophages arrested in G1 phase [4]. Furthermore, Fc-mediated phagocytosis is up-regulated in S phase, consistent with an increased expression level of Fc receptors [5,6].

Phagocytosis is an important host defence mechanism. Macrophages protect the host by ingesting microbes and killing them. Macrophages also function as antigen-presenting cells in regulating the function of lymphocytes. Each of these processes begins with the macrophage binding to microbes by a cell surface receptor. Two of the major macrophage receptors are Fcγ receptors (FcγR) and complement receptors (CR), which bind with antibody-opsonized and complement-opsonized microbes, respectively [7,8].

It is commonly believed that dead and damaged macrophages are replenished by an influx of blood monocytes that differentiate into macrophages in tissue [9]. However, there is increasing evidence that tissue macrophages can also be renewed by local proliferation [10–15]. Interestingly, tissue macrophages were found to replicate upon the stimulation of colony-stimulating factors, especially during inflammation [16]. These findings suggest that local proliferation of macrophages in infected tissue could provide an alternative mechanism for the replenishment of tissue macrophages.

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In the present study, we investigated FcγR and CR expression on macrophage-like cells as a function of cell cycle. We confirmed the prior observation made by Walkers *et al.* [5,6] that FcγR expression on macrophage-like cell line was increased as the cells progressed from G1 to G2 phases. Moreover, we found that CR3 expression varies as a function of cell cycle in a manner similar to FcγR. Analysis of cell size and receptor expression suggests that FcγR and CR3 expression on macrophages are determined largely by cell size enlargement during cell cycle. Furthermore, the study on phagocytosis of live and dead *Cryptococcus neoformans* (Cn) as well as Cn strains with different virulence revealed interesting differences.

Materials and methods

Cell lines

The macrophage-like cell line J774·16 was used for all studies. This cell line has phenotypic characteristics similar to murine peritoneal macrophages [17]. Furthermore, we have demonstrated recently that all effects of phagocytosis on cell cycle observed with J774·16 cells could be reproduced in primary murine macrophages [18]. Cells were cultured at 37°C with 10% CO_2 in Dulbecco's Modified Eagle's Media (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 10% NCTC-109 medium and 1% non-essential amino acids.

Yeast strains

Cn strain 24067 (serotype D) was obtained from the American Type Culture Collection (Manassas, VA, USA). Strain H99 was obtained from Dr John Perfect (Durham, NC, USA). Strain RC-2 is a variant of Cn strain 24067 [19]. The smooth (SM) parent strain RC-2 generates mucoid (MC) colony phenotype variants which are more virulent [20]. Cn was cultured for 2–3 days in Sabouraud dextrose broth at 30°C with moderate shaking at 150 r.p.m. Cells were collected by centrifugation, washed with phosphate-buffered saline (PBS) three times and counted in a haemocytometer. Heat-killed Cn were prepared by incubating cultures in a water bath at 65°C for 30 min. Cultures were plated for colony-forming units (CFU) to verify that cell killing occurred.

Centrifugal elutriation

Counterflow centrifugal elutriation (CCE) is a method for isolating cellular subpopulations on the basis of their sedimentation coefficient, itself a function of cell volume and density. Confluent (90%) J774·16 cells were harvested from two 750 ml cell culture flasks with PBS supplemented with 0·1% bovine serum albumin (BSA) and 1 mM Na ethylenediamine tetraacetic acid (EDTA) and elutriated in DME. The cell suspension of $1{\cdot}5 \times 10^8$ cells was loaded at 20 ml/min into a rotating elutriator rotor (Beckman JE-5·0 in a Beckman J-6B centrifuge, Beckman Instruments Inc., Palo Alto, CA, USA) while the rotor speed was kept constant at 3500 r.p.m. Cells were collected in 100 ml fractions at increasing flow rates using a peristaltic pump. The flow rates were 52 ml/min for the first fraction, 56, 65, 75, 80 and 85 ml/min for fractions 2–6, respectively, and the rest of the cells for the fraction 7. Cells were then collected by centrifugation at 400 ***g*** for 5 min and resuspended in DME for further experiments.

Cell size measurement

The cells in elutriation fractions were wet mounted on slides and photographed at 40×. For each slide, 10 fields were taken. The cell diameter was then measured by Photoshop (Adobe, San Jose, CA, USA). If the cells were not round-shaped, both the long and short diameters were measured and averaged. At least 30 cells were measured in each fraction and the diameters of these cells were averaged. The cell surface area and the whole cell volume were calculated by equations $S = \pi r^2$ and $V = 4/3\ \pi r^3$, respectively (S = area, V = volume, r = radius = 1/2 diameter).

Cell staining

For cell staining of unelutriated cells, J774·16 cells were cultured in six-well plates to a density of 1×10^6 cells per well. The cell monolayer in each well was harvested by incubating cells with PBS for 10 min in room temperature and gently pipeting [21]. The floating cells were transferred to a 1·5-ml tube. For cell staining of elutriated J774 cells, elutriated fractions were transferred to 1·5 ml tubes as 1×10^6 cells per tube. After centrifuge, the cells were resuspended and fixed with 1 ml 3·7% formaldehyde in PBS for 7 min. The cells in the tubes were washed once with 1 ml PBS and once with 1 ml staining buffer (2% FCS with PBS) and resuspended with 1 ml staining buffer before staining. For cell surface receptor staining, fluorescein isothiocyanate (FITC)-conjugated monoclonal rat antibody to murine CD16/32 (FcγRIII/II; BD Pharmingen, San Diego, CA, USA) was used (1 μg/ml) to detect FcγR expression [22]. FITC-conjugated monoclonal rat anti-mouse antibody to CD11b (CR3; BD Pharmingen) and hamster antimouse antibody CD11c (CR4; BD Pharmingen) were added (0·25 μg/ml and 1 μg/ml, respectively). Irrelevant IgG was used as a negative control. The tubes were then incubated at room temperature for 30 min. After incubation, the cells were washed three times with PBS to remove non-bound antibody and kept in the dark at 4°C until they were analysed. For whole cell receptor staining, cells were permeabilized by suspending in a 0·2% Triton X 100 in PBS for 3 min before immunostaining.

Phagocytosis assay

Elutriated fractions of macrophages were replated into six-well plates to a density of 1×10^6 cells per well in DMEM with 0·5% heat-inactivated FCS. Phagocytosis assays were

performed after 2 h when the seeded cells were attached to the bottom of the plates. In Fc-mediated phagocytosis assays, live or heat-killed Cn cells of strain 24067, or Cn RC-2 strain SM or MC were added at an effector to target ratio of 1 : 1. FITC-conjugated Cn capsule-specific monoclonal antibody (mAb), 18B7, was used as an opsonin at 10 μg/ml. Incubation was carried out in 10% CO_2 at 37°C. In complement-mediated phagocytosis assays, FITC-labelled Cn strain H99 was added at an effector to target ratio of 1 : 1 and 20% guinea pig serum (Calbiochem, CA, USA) was added to promote phagocytosis. After incubating for 1·5 h, by which the maximal percentage of phagocytosis was reached in this condition, any remaining extracellular yeast cells were removed with three washes of PBS. The resulting monolayer was resuspended with PBS and fixed with ice-cold 70% ethanol overnight at 4°C.

Flow-cytometric analysis [fluorescence activated cell sorter (FACS)]

After staining cells with the FITC-conjugated antibodies, cell pellets were washed and fixed as indicated above. For DNA staining, the cells were incubated at room temperature for 30 min with 20 μg/ml propidium iodide (PI; Molecular Probes, Eugene, OR, USA) and RNase at a final concentration of 200 μg/ml in PBS. The samples were then analysed by FACScan (Becton-Dickinson, Mountain View, CA, USA) and the flow cytometry data were analysed by ModFit 3·0 software (Verity Software House, Topsham, ME, USA) and Flowjo software (Tree Star, Ashland, OR, USA) for cell cycle distribution and FITC staining of cells. FACS was also used to determine the phagocytosis percentage, which is defined as the percentage of macrophages with internalized particles. After phagocycytosis, the cells were prepared as described above. The proportion of yeast in the intracellular and extracellular compartments was determined by quenching extracellular FITC dye conjugated to Cn with 2 mg/ml crystal violet prior to the FACS analysis [23]. The samples were analysed by FACScan for the number of cells with FITC signal (phagocytosed) and without FITC signal (unphagocytosed). The phagocytosis percentage was calculated as number of FITC positive cells × 100/number of total cells.

Statistics

Comparison between multiple groups was performed by analysis of variance (ANOVA) using Excel; $P < 0{\cdot}05$ was considered significant.

Results

FcγR and CR3 expression varied as a function of cell cycle

J774 cells were stained with antibodies to CD16/32, CD11b and CD11c conjugated to FITC and then counterstained with PI. The proportion and the number of cells in the different cell cycle phases were identified by PI staining using FACS (Fig. 1a). The relative intensities of FITC staining on the cell surface and whole cell were measured by FACS (Fig. 1b). Cells in the G1, S and G2 cell cycle phases were gated by the analysis Flowjo software. For each cell cycle phase, the cell surface and whole cell expressions of FcγR and CRs were determined from the relative intensity of the fluorescence staining (Fig. 1c–e). Cells in each cell cycle phase expressed FcγR (CD16/32) and CR3 (CD11b) (Fig. 1). None of the J774 cells expressed CR4 (CD11c) (data not shown), confirming a previous study [24]. The surface and whole cell expression level of both FcγR and CR3 increased during cell progression from G1 to G2 (Fig. 1f–g). There was a 50–60% increase of FcγR and CR3 expression from G1 to G2, as indicated by the intensity of FITC binding to the cell surface or the whole cell level.

Macrophage cell diameter increased as cells progressed from G1 to G2 phases

Exponentially growing J774 cells were subjected to centrifugal elutriation (Fig. 2). The cells were separated into seven fractions depending on their cell sizes. Microscopic evaluation of the material in each fraction revealed that fraction 1 contained a significant amount of cell debris, and thus was discarded. The diameter of cells in the remaining fractions was measured under microscope. The size of J774 cells in suspension ranged from 10 μm to 16·5 μm in diameter, and increased with each subsequent fraction. The majority of cells (around 60%) elutriated in fractions 2 and 3 were mainly in G1 phase and the percentage of cells in G1 phase decreased in each of the following fractions. The cells in S phases appeared first in fraction 4 and reached plateau in fractions 5 and 6. In fraction 7, the cells in S phase decreased. The cells in G2 phase appeared first in fraction 5 and increased in fractions 6 and 7 (Fig. 2c). The proportions of cells in G1 phase in each fraction were measured by Modfit and were correlated with measured cell diameter (Fig. 2d). The result showed that cell diameter increased with the decrease of G1 percentage. However, the increase was not linear, especially for the cells in G1 phase, which had increased size before they went into S phase.

FcγR and CR3 expression increased linearly with cell size enlargement during cell cycle

Elutriated fractions were stained with FITC-conjugated anti-CD16/32 and CD11b before and after cell permeabilization to determine the relative amounts of surface and whole cell receptor expression FcγR and CR3, respectively. The intensity of FITC signals was then correlated with cell size parameters calculated previously (Fig. 3). The surface receptor expression (Fig. 3a,b) was correlated positively with cell surface area and the whole cell receptor

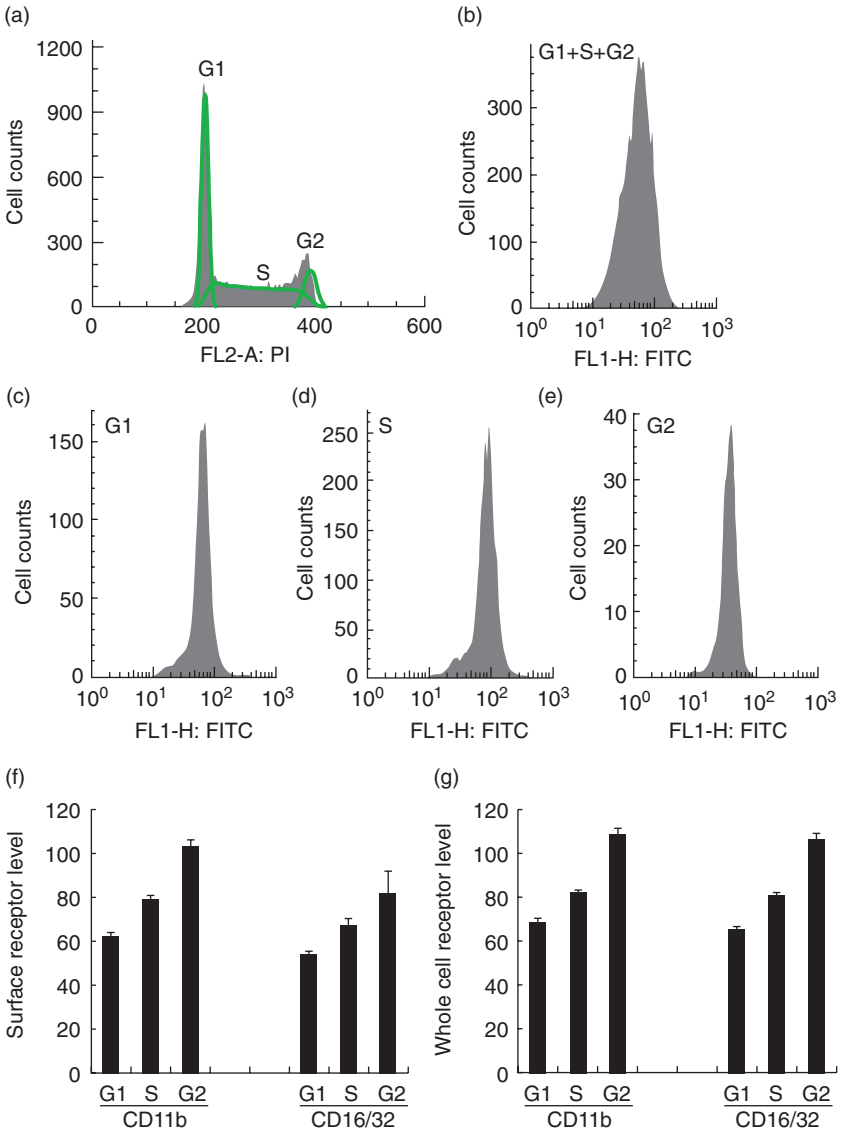

Fig. 1. Fcγ receptor (FcγR) and complement receptor (CR)3 expression of J774 cells as a function of cell cycle. (a) Cell cycle analysis of unelutriated J774 cells. J774 cells were stained by fluorescein isothiocyanate (FITC) conjugated antibodies to CD16/32, CD11b or CD11c and counterstained by propidium iodide (PI). The cell cycle distribution was analysed by the Flowjo software according to the PI content in cells. The green lines showed the curve-fitting of cell cycle phases by the Flowjo software. (b–e) The *x*-axis showed CD11b staining (FITC) as an example. The results for CD16/32 staining were similar [both data showed in (f) and (g)]. CD11c staining was negative for the cells in all the cell cycle stages (data not shown). (b) Shows FITC histogram of all the tested cells (G1 + S + G2) in (a). (c,d,e) Cells located in each cell cycle phase (G1, S and G2) were gated by the Flowjo software and FITC histogram of these cells in various cell cycle phases were reported. (f,g) Surface expression (f) and whole cell expression (g) of FcγR and CR3 of J774 cells with G1, S and G2 phases. Comparison of receptor expression level of G1, S and G2 revealed significantly differences $P < 0{\cdot}01$. Each bar was an average of fluorescent staining measurements in three independent experiments and the brackets denote standard deviation. The different *y*-axis scales were automatically generated by Flowjo software based on the magnitude of the peak.

expression was correlated positively with whole cell volume (Fig. 3c,d).

Phagocytosis efficacy did not correlate with FcγR and CR3 surface expression through cell cycle progression

Macrophages of elutriated fractions were analysed for their ability to phagocytose live or heat-killed Cn. The phagocytosis percentage was measured by FACS and plotted *versus* surface expression of FcγR and CR3, as determined by immunostaining (Fig. 4). Macrophages ingested considerably more dead Cn than live Cn. A dissociation of FcγR (Fig. 4a) and CR3 (Fig. 4b) surface expression level with phagocytosis efficacy was observed in phagocytosis of both live Cn and dead Cn. Interestingly, the phagocytosis efficacy of dead Cn, decreased linearly when plotted *versus* FcγR and CR3 surface expression while the phagocytosis of live Cn showed a different biphasic pattern, being higher in G1 and G2 phases and lower in S phase.

Correlation of cell cycle distribution and phagocytosis efficacy were different between phagocytosis of live and dead Cn when cells exited G1 phase

The efficacy of phagocytosis as a function of the percentage of cells in G1, S and G2 revealed different curves for live and dead yeasts opsonized with IgG1 antibody or complement (Fig. 5). Both IgG1 and complement-mediated phagocytosis as a function of cell cycle yielded similar curves except that the complement-mediated phagocytosis had lower phagocytosis efficacy compared to that of Fc-mediated phagocytosis. For the cells in G1 phase (G1 = 100%, S = 0, G2 = 0), phagocytosis efficacy of both live and dead Cn dropped significantly when the majority of cells approached interface of

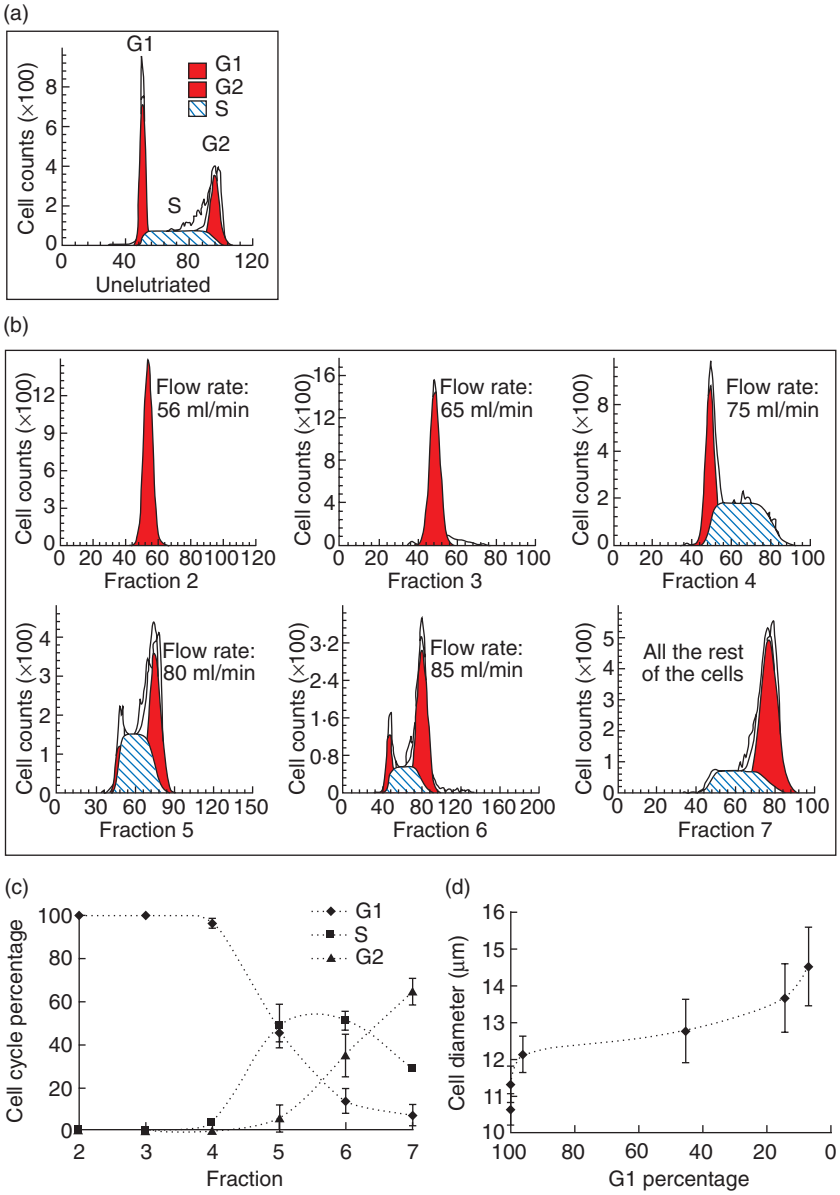

Fig. 2. Cell cycle analysis and cell diameter measurement of elutriated J774 cells. (a) Cell cycle analysis of J774 cells before elutriation. The cells were stained with propidium iodide (PI). The cell cycle distribution was analysed by the Modfit 3·0 software according to the PI content in cells. The red colour-filled area (G1 or G2 phase) and hatched area (S phase) shows the curve-fitting of cell cycle phases by the Modfit 3·0 software. (b) Cell cycle analysis of elutriated fractions of J774 cells. The cells were separated into six fractions according to cell size (the elutriation flow rate is indicated for each elutriation fraction) and then the elutriated fractions were analysed as described in (a). The different *y*-axis scales were automatically generated by Modfit software based on the magnitude of the peak. (c) G1, S and G2 distribution of elutriated fractions. Each point was an average of cell cycle percentages measured in four independent experiments and the brackets denote the standard deviation. (d) Correlation of G1 percentage of elutriated fractions and measured cell diameters. Each point was an average of cell diameter measurements in four independent experiments and in each experiment at least 30 cells randomly chosen from the elutriated fractions were measured. The brackets denoted standard deviation.

G1–S. When the cells exited G1 phase and went into S and G2 phase, correlation of cell cycle and phagocytosis efficacy of live Cn and dead Cn revealed significant differences. The phagocytosis efficacy of live Cn increased as the percentage of cells in G1 decreased, and the percentage of cells in G2 increased (Fig. 5a,c). No clear relationship could be drawn between the percentage of cells in S phase and phagocytosis efficacy (Fig. 5b). As for phagocytosis of dead Cn, phagocytosis efficacy changed little with the cell cycle distribution when cells exited G1 phase (Fig. 5d–f).

Correlation of cell cycle distribution and phagocytosis efficacy were different between Cn strains with different virulence

Macrophages of elutriated fractions were analysed for their ability to phagocytose live Cn strain variants differing in virulence (SM and MC) via Fc-mediated pathway. The MC variant is more virulent [20]. The phagocytosis percentage was measured by FACS and plotted *versus* surface expression of FcγR determined by immunostaining (Fig. 6). Similar results to those described above were observed. Phagocytosis of SM was more efficient than phagocytosis of MC, especially when macrophages were in G1 phase. Most interestingly, we found that the difference of phagocytosis efficacy was predominately caused by differences in phagocytosis during G1 phase. Once macrophages exited G1, the difference of phagocytosis efficacy diminished.

Discussion

A study conducted almost two decades ago [5] showed that FcγR expression per cell varied as a function of cell cycle, and concluded that this variation was largely a consequence of

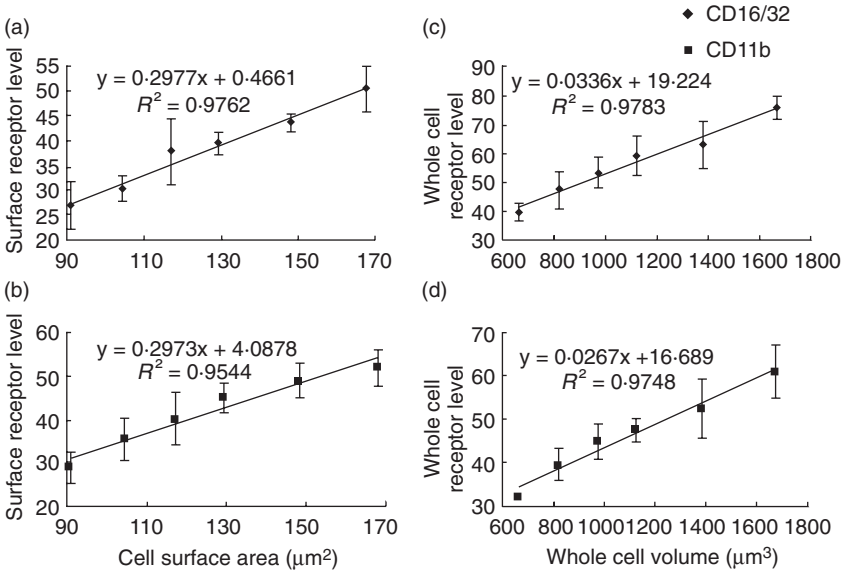

Fig. 3. Correlation of cell sizes and Fcγ receptor (FcγR) and complement receptor (CR)3 expression for cells in the elutriated fractions. Receptor level was measured as the relative intensity by FACS. (a,b) Correlation of cell surface areas calculated from cell diameters and surface receptor expression of elutriated fractions. (c,d) Correlation of whole cell volumes calculated from cell diameters and whole cell receptor expression of elutriated fractions. Each point was an average of fluorescent staining measurements of four independent experiments and the brackets denoted standard deviation.

the cell size changes that accompanied replication. That study relied on the binding of radiolabelled IgG2a to elutriated cells differing in cell cycle state and calculated receptor density by Scatchard analysis. They concluded that the higher FcγR density in G2 cells would make them more avidly phagocytic, although subsequent studies have shown this supposition not to be the case. In the years since Gandour and Walker [5] carried out their pioneering study, a variety of FcγR has been described that binds preferentially to the different antibody isotypes. For example, the IgG2a isotype used in the previous study binds preferentially to FcγRI, which could have conceivably affected their conclusions if the expression of the various FcγRs is regulated differentially. Given the increasing evidence linking phagocytosis and cell cycle and the apparent discrepancy between the reported maximal FcγR expression in cell cycle and phagocytosis, we revisited this question with a different methodology and extended the study to a receptor of the innate immune system, CR3.

The J774·16 macrophage-like cell line was chosen because it is a standard cell line used in phagocytosis studies that faithfully mimics many aspects of macrophage function. In this regard, Gandour and Walker [5] had also relied on the macrophage-like cell line P388D1 for their study. Although one must be cautious in extrapolating results from cell lines to primary cells, a study using primary cells is not feasible because these cells are overwhelming in the G1 state and the frequency of cell cycle progression is relatively low [6,18]. In our study, both FcγR and CR3 were expressed in all cells at all phases of the cell cycle. Analysis of both surface and total cell

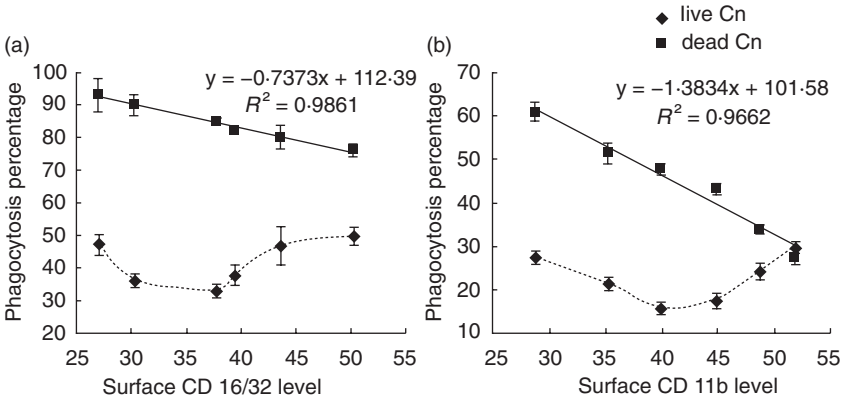

Fig. 4. Correlation of surface receptor expression level and phagocytosis of live and heat-killed *Cryptococcus neoformans* (Cn). Receptor level was measured as the relative intensity by fluorescence activated cell sorter (FACS). (a) Correlation of surface CD16/32 receptor expression level and phagocytosis of live and heat-killed Cn via Fc-mediated (18B7) pathway. (b) Correlation of surface CD11b receptor expression level and phagocytosis of live and heat-killed Cn via complement-mediated (guinea pig serum) pathway. Each point is an average of phagocytosis percentage measurements in three independent experiments and the brackets denoted standard deviation. Please note that the dash lines for phagocytosis of live Cn were not regression lines.

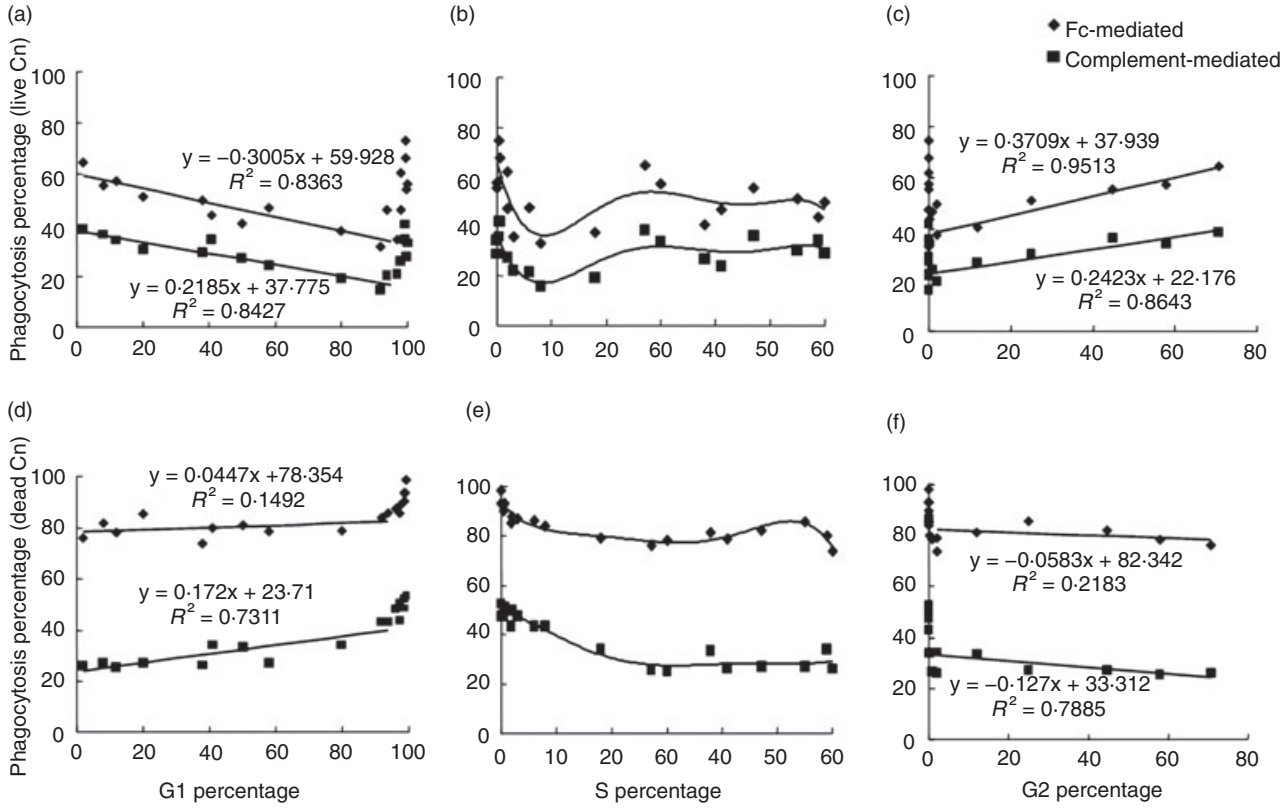

Fig. 5. Correlation of cell cycle distribution and phagocytosis of live and heat-killed *Cryptococcus neoformans* (Cn). (a–c) Correlation of G1 percentage (a), S percentage (b) and G2 percentage (c), with phagocytosis of live Cn via Fc-mediated [monoclonal antibody (mAb) 18B7] pathway and complement-mediated (guinea pig serum) pathway. (d–f) Correlation of G1 percentage (d), S percentage (e) and G2 percentage (f), with phagocytosis of live Cn via Fc-mediated (mAb 18B7) pathway and complement-mediated (guinea pig serum) pathway. Each point is a phagocytosis percentage measurement and all the three independent elutriations were pooled together. G1 cells (G1 = 100%, S = 0, G2 = 0) were excluded for all the equation fittings. (a, c, d, f) Linear fitting was used. Polynomial fitting (X^4) was used for (b) and (e).

FcγR expression as a function of cell cycle confirmed the observation by Gandour and Walker [5] that expression increases as cell cycle progresses from G1 to G2. The fact that similar results have now been observed with two different cell lines using different methods increases confidence in the biological significance of this phenomenon. FcγR expression was correlated strongly with cell size consistent with the notion that the measured increase in receptor expression was a result of larger cells. Similar results were obtained for CR3, implying that neither the phagocytic receptors of the innate and adaptive immune system were regulated differentially during the cell cycle stages.

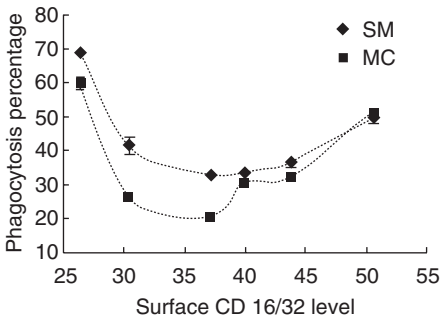

Fig. 6. Correlation of surface CD16/32 receptor expression level and phagocytosis of *Cryptococcus neoformans* (Cn) strains SM and MC with different virulence via Fc-mediated phagocytosis. Each point is the average of three independent experiments and the brackets denote standard deviation.

Although FcγR and CR surface level were thought to be major determinants of phagocytosis efficacy of Fc- and complement-mediated phagocytosis process our results indicate a more complex relationship. The finding that FcγR and CR3 expression peaks for G2 cells, while the efficacy of phagocytosis varied with cell cycle depending on whether one used live or dead Cn as well as Cn strains with different virulence, implies a dissociation between FcγR and CR3 expression and phagocytic efficacy. Although this study did not investigate the cellular basis for difference of phagocytic efficacy during cell cycle phases our results suggest strongly that this phenomenon is not related to phagocytic receptor density and probably reflects other cellular processes. In fact, our results could be interpreted to suggest the existence of mechanisms that reduce phagocytosis for the cells at the

G1–S junction, as such cells have initiated DNA duplication machinery and are in a state that could make them more vulnerable to damage by ingesting microbes. Studies to investigate the mechanisms responsible for differences in phagocytic efficacy during cell cycle stages should focus on processes other than phagocytic receptor expression, especially for G1 cells. In addition, the results raise the tantalizing suggestion that live Cn might be able to target macrophages in G2 phase which are ready to replicate and therefore to co-opt the replicating system of macrophages to disseminate the infection, a hypothesis proposed in our previous published study [18].

Lastly, we note that dead Cn and Cn strains with lower virulence were ingested more readily than live Cn and Cn strains with higher virulence by phagocytosis, a result implying that live Cn and Cn strains with higher virulence interfere actively with the process of phagocytosis. A mechanism by which live Cn may interfere with phagocytosis is the active release of polysaccharide, which is known to inhibit phagocytosis [25]. Furthermore, live Cn may also produce an anti-phagocytic protein that could reduce the efficiency of this process [26]. Hence, the efficacy of phagocytosis in this system appears to be a function of both the viability and virulence of the yeast cell and the cell cycle stage of the macrophage-like cell.

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