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Quantification and Characterization of Phagocytosis in the Soil Amoeba *Acanthamoeba castellanii* by Flow Cytometry

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Phagocytosis in the common grazing soil amoeba *Acanthamoeba castellanii* was characterized by flow cytometry. Uptake of fluorescently labelled latex microbeads by cells was quantified by appropriate setting of thresholds on light scatter channels and, subsequently, on fluorescence histograms. Confocal laser scanning microscopy was used to verify the effectiveness of sodium azide as a control for distinguishing between cell surface binding and internalization of beads. It was found that binding of beads at the cell surface was complete within 5 min and 80% of cells had beads associated with them after 10 min. However, the total number of phagocytosed beads continued to rise up to 2 h. The prolonged increase in numbers of beads phagocytosed was due to cell populations containing increasing numbers of beads peaking at increasing time intervals from the onset of phagocytosis. Fine adjustment of thresholds on light scatter channels was used to fractionate cells according to cell volume (cell cycle stage). Phagocytotic activity was approximately threefold higher in the largest (oldest) than in the smallest (newly divided) cells of *A. castellanii* and showed some evidence of periodicity. At no stage in the cell cycle did phagocytosis cease. Binding and phagocytosis of beads were also markedly influenced by culture age and rate of rotary agitation of cell suspensions. Saturation of phagocytosis (per cell) at increasing bead or decreasing cell concentrations occurred at bead/cell ratios exceeding 10:1. This was probably a result of a limitation of the vacuolar uptake system of *A. castellanii*, as no saturation of bead binding was evident. The advantages of flow cytometry for characterization of phagocytosis at the single-cell level in heterogeneous protozoal populations and the significance of the present results are discussed.

Certain protozoa, e.g., *Acanthamoeba castellanii*, appear to be entirely dependent on endocytotic processes (phagocytosis and pinocytosis) to fulfill their nutritional requirements in the natural environment (34). Furthermore, growth of protozoa in liquid culture under laboratory conditions may be limited in the absence of particles (e.g., those produced during autoclaving of certain media) that stimulate endocytosis (40). In recent years, with improvements in sampling and analytical techniques, the prevalence of protozoa in the natural environment and the ecological importance of protozoal phagocytosis have become recognized fully. For example, grazing by heterotrophic protozoa has now been accepted as the most important factor responsible for decreases in bacterial numbers in aquatic ecosystems (24, 42, 47). Protozoal grazing also greatly enhances remineralization and recycling of nutrients within aquatic ecosystems and transfer of bacterial productivity to higher trophic levels (6, 8, 42, 47). Protozoa, particularly amoebae, are also recognized as major regulators of bacterial populations in soil (1, 25, 52). Naked amoebae alone may account for more than 60% of observed decreases in bacterial numbers in the field (15). Algae, detritus, and fungal spores and hyphae are also highly susceptible to phagocytosis by protozoa (16). Thus, amoebae enhance nutrient recycling in soils out of proportion to their own biomass and increase nutrient availability to other organisms, e.g., microorganisms, nematodes, and plants (7, 52). Protozoal grazing has also been implicated in the control of bacterial numbers during water purification and sewage treatment processes (16), and certain evidence suggests that bioremediation processes involving bacteria may become strongly inhibited in the presence of phagotrophic protozoa (55). A potential application of amoebae in the control of toxic cyanobacterial blooms in aquatic ecosystems has also been suggested (22).

Ingested bacteria may be resistant to the normal intravacuolar digestive mechanism of certain amoebae, e.g., *A. castellanii*, and here, vacuolar disruption results in release of bacteria into the cytoplasm, where they may proliferate as endosymbionts (26) or parasites (21, 39) to the host. Such interactions are of particular concern where they involve coliforms, *Legionella* spp., or other human pathogens, as it has been demonstrated that these organisms are far more resistant to treatments used to control their multiplication (e.g., chlorine application) when associated with a protozoan host than when free-living (32, 33). Protozoal phagocytosis may thus increase the likelihood of exposure of humans to pathogenic bacteria. In addition, the proliferation of pathogenic *Acanthamoeba* spp. has been linked to the presence of digestible bacteria in contact lens solutions; thus, phagocytotic events have been implicated as the first steps in the pathogenesis of amoeba-induced keratitis in humans (9).

The soil amoeba *A. castellanii* also displays avid phagocytotic activity in liquid culture medium when challenged with particles, e.g., live or dead bacteria and yeasts or latex beads (4, 18, 23, 38), thus facilitating studies of phagocytosis at the biochemical level. Phagocytosis in *A. castellanii* is now known to be associated with extracellular release of AMP (23), a respiratory burst which may be necessary for the generation of reactive oxygen metabolites during subsequent digestion (18), and with enhanced degradation of phosphatidylinositol bisphosphate to inositol 1,4,5-trisphosphate (2); a connection of phagocytosis with second messenger pathways in *A. castellanii* is an implication of the study reported in reference 2. Furthermore, recent evidence has indicated that increases in the unsaturation

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of membrane fatty acids, following chilling of *A. castellanii*, coincide with increases in phagocytotic activity, suggesting a role of plasma membrane fluidity in the control of phagocytosis (4).

The interest that has been generated in and the ecological significance of protozoal phagocytosis highlight the need for a detailed understanding of the process. However, to date such studies with protozoa have been limited. This may be largely attributable to the laborious and/or inaccurate techniques that have been employed for analysis of phagocytosis. Many studies have relied on light-microscopic counts. However, in view of the large degree of variability of particle uptake in individual cells (43), in addition to the difficulty of discerning more than 12 particles in one cell and the time required to count large numbers of cells by eye (53), such data can only be regarded as semiquantitative at best. Counting of particles remaining in media after phagocytosis overcomes the problem of counting individual amoebae but does not allow discrimination between bound and internalized (phagocytosed) particles (23). Spectrophotometric studies of phagocytosis of latex beads, by dissolution of phagocytosed particles in dioxane, have proved useful for the examination of uptake levels in whole populations but suffer the disadvantage that they yield no information on bead uptake in individual cells (11, 12).

Flow cytometry has proved to be an increasingly valuable technique in recent years for rapid discrimination between microbial populations which display heterogeneity with respect to properties of light scatter or fluorescence (37). The method allows the counting of large numbers of cells, with associated increases in statistical significance. Such studies have been extended to protozoan grazing of photosynthetic microorganisms in aquatic ecosystems, where phagocytosed organisms can be discriminated according to their fluorescent-pigment composition (17). Furthermore, Steinkamp et al. (49) have described a flow-cytometric technique that allows rapid analysis of phagocytosis of fluorescently labelled latex beads by pulmonary macrophages, and this has been extended to the examination of rates of particle uptake in protozoa (27).

The purpose of the present study was to present a detailed flow-cytometric characterization of phagocytosis in *A. castellanii*. We demonstrate high degrees of variability in phagocytotic activity between whole populations of *A. castellanii* incubated under differing conditions and also between individual cells. Fractionation of populations according to cell volume was used to demonstrate the cell cycle dependence of phagocytosis, while control sodium azide incubations differentiated between bound and internalized particles. The results highlight the applicability of flow cytometry for phagocytosis research with amoebae.

MATERIALS AND METHODS

Organism, medium, and growth conditions. *A. castellanii* was grown in PGY medium, containing (percent [wt/vol]) Difco Proteose Peptone (0.75), Oxoid yeast extract (0.75), and glucose (1.5). Cultures (200 ml in 500-ml Erlenmeyer flasks) were inoculated with cells from 48-h cultures to give an initial cell density of approximately 2×10^5 ml^{-1} and were incubated at 30°C with rotary aeration at 200 rpm. For growth curves, cell numbers were determined with a Fuchs-Rosenthal hemocytometer slide, after appropriate dilution with distilled water; more than 800 cells were counted in each sample.

Phagocytosis assay. Undiluted samples (0.9 ml) from 48-h cultures (unless stated otherwise) were transferred to 1.5-ml microcentrifuge tubes and incubated with shaking at 200 rpm (unless stated otherwise) at 30°C, in either the presence or the absence of 12.5 mM sodium azide. After 5 min of equilibration, 100 μl of fluorescently labelled yellow-green latex microbeads (diameter, 1.0 μm; Polysciences, Warrington, Pa.) were added to the tubes to a final density of approximately 5×10^7 beads ml^{-1} (unless stated otherwise). Parallel incubations in the presence or absence of sodium azide always had the same bead/cell ratio. After 10 min of incubation (unless stated otherwise) with latex beads, phagocytosis was stopped by dilution of the cell-plus-bead suspension to 10 ml with buffer (100 mM mannitol–15 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.2, adjusted with 2 M NaOH) at 4°C. Cells were separated from free beads by centrifugation for 1 min at 750 × *g*. The supernatant, which contained >95% of all free beads, was discarded. The cell pellet was suspended in 1 ml of buffer and maintained at 4°C until analysis. All samples were analyzed within 24 h of experiments; control experiments confirmed that no change in total numbers of phagocytosed or cell surface-bound beads occurred during storage of washed cells at 4°C for up to 1 week.

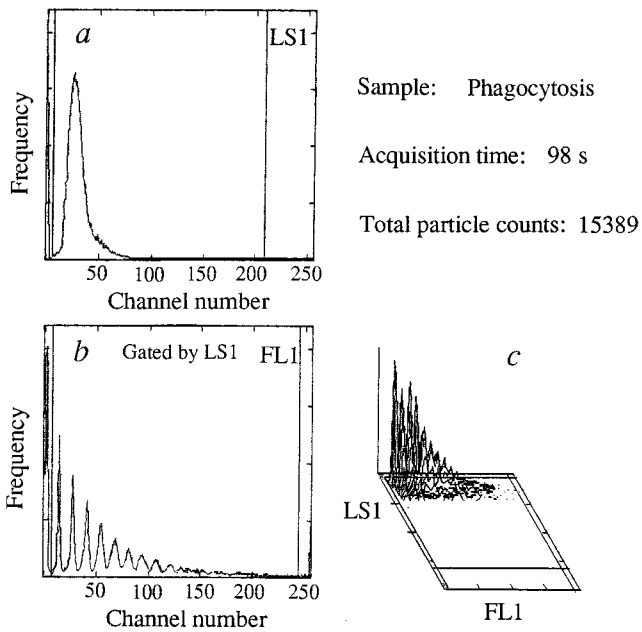

FIG. 1. Forward-angle light scatter (a), fluorescence (b), and light scatter-versus-fluorescence (c) histograms of flow-cytometric data collected for *A. castellanii* containing fluorescently labelled latex beads.

Flow cytometry. Cells were analyzed for fluorescent beads by a flow-cytometric method adapted from that of Steinkamp et al. (49). Sample analysis was performed with a Skatron Argus flow cytometer (Skatron, Tranby, Norway). Photomultiplier voltages were 200 and 350 V and gain settings were 1.0 and 4.0 for forward-angle light scatter and fluorescence, respectively. Green fluorescence signals were collected with an Argus B1 filter block. Thresholds were set on forward light scatter channels so that only data for cell-associated fluorescence were presented in fluorescence histograms; noise and fluorescence from residual free beads that had not been taken up by the cells were not recorded. Typical histograms (Fig. 1) contained data from approximately 15,000 cells, measured at a flow rate of 2 or 5 μl min^{-1}. The proportions of cells that had taken up fluorescent beads, as well as numbers of cells under each peak of fluorescence (the intensity of fluorescence of each peak being linearly related to the number of beads taken up), were obtained by appropriate adjustment of cursors on fluorescence channels. The latter values allowed calculation of the total number of beads taken up by a sample of cells. Appropriate adjustment of thresholds on light scatter channels during particle collection allowed analysis of phagocytosis in fractions of cell populations differentiated by cell volume. The average number of beads bound to the cell surface was obtained from fluorescence histograms of cells incubated in the presence of sodium azide. These control values were subtracted from those obtained for cells incubated in the absence of sodium azide to give the average number of internalized (phagocytosed) beads. For clarity, bead uptake is normalized to the average per 100 cells throughout this paper.

Confocal laser scanning microscopy. A Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, Calif.) was used to observe fluorescently labelled latex beads in *A. castellanii*, with appropriate excitation and barrier filters. Observations were performed with a 60× oil immersion lens. Neutral density filters were used to attenuate the laser intensity. A Silicon Graphics work station was used to prepare confocal laser scanning microscopic images; prints were produced directly from the computer model.

RESULTS

Effect of sodium azide on uptake of latex beads by *A. castellanii*. Previous investigations of phagocytosis in *A. castellanii* cells and in phagocytic mammalian cells have demonstrated

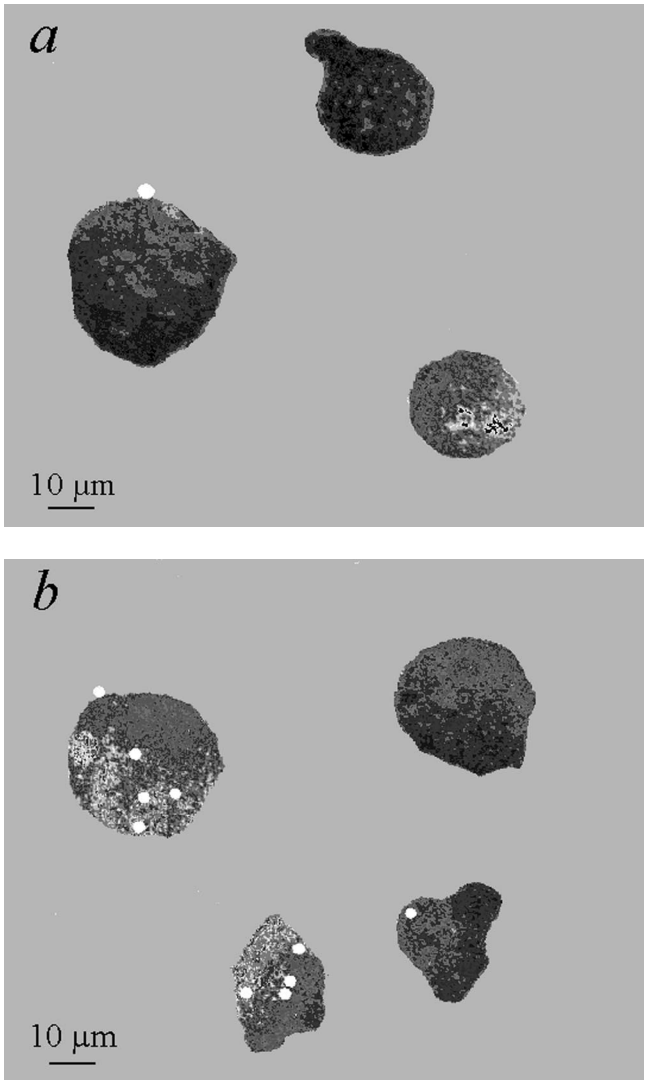

FIG. 2. Confocal laser scanning images of *A. castellanii* cells with fluorescent latex beads. Cells were incubated with 1-μm diameter fluorescently labelled latex beads in either the presence (a) or the absence (b) of 12.5 mM sodium azide. Images are of 0.5-μm optical sections through intact cells and were prepared with a Silicon Graphics work station.

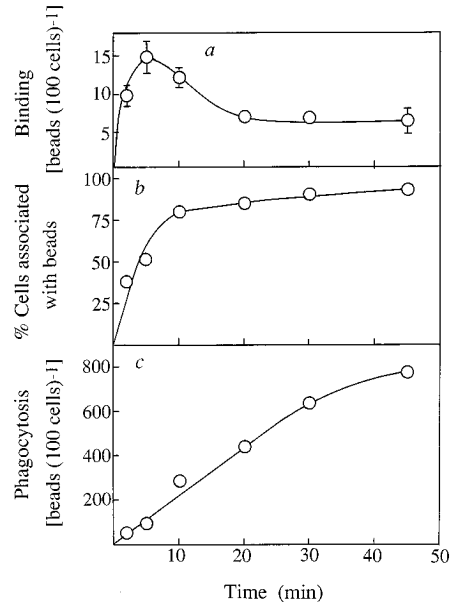

FIG. 3. Time course of bead uptake. Cells were incubated in growth medium at 30°C, in either the presence or the absence of 12.5 mM sodium azide, for varying periods of time after the addition of latex beads (to approximately 5×10^7 ml^{-1}). (a) Numbers of beads bound at the cell surface. (b) Proportions of cells associated with beads (bound or phagocytosed). (c) Total numbers of beads phagocytosed. Points are averages from three replicate samples, each representing data collected from at least 15,000 cells, ± standard errors of the mean (error bars) where these values exceed the dimensions of the symbols.

that sodium azide inhibits internalization of particles without affecting the binding phase of uptake (3, 19, 49, 53). In the present study, sodium azide (12.5 mM) was found to be an effective control for determination of numbers of beads associated with the cell surface of *A. castellanii*. Bead uptake in cells incubated at 30°C in the presence of sodium azide was comparable to and never greater than that in cells incubated at 4°C without sodium azide. Figure 2 shows typical confocal laser scanning microscope images of *A. castellanii*, previously incubated for 20 min with fluorescently labelled latex beads in either the presence (Fig. 2a) or the absence (Fig. 2b) of 12.5 mM sodium azide. As the images represent 0.5-μm optical sections through intact cells, the method distinguishes clearly between bound and internalized beads. By conventional fluorescence-microscopic techniques, beads located at differing depths in the cells are observed simultaneously and consequently only those beads bound at the greatest circumference perpendicular to the optical axis will be distinguishable from internalized beads. Beads were observed only at the surface of cells incubated in the presence of azide, whereas beads were observable both intracellularly and bound at the surface of phagocytosing cells incubated in the absence of azide. Examination of a large number of fields of view confirmed that approximately equal numbers (shown as means ± standard errors of the mean) of beads were bound to the surface of cells incubated in the presence (7.6 ± 0.3 beads 100 cells^{-1}) or absence (7.2 ± 0.4 beads 100 cells^{-1}) of sodium azide, whereas beads were internalized only in the latter case.

Time course of phagocytosis by *A. castellanii* and analysis of bead numbers in individual cells. Phagocytosis by *A. castellanii* was examined over a time course of 45 min (Fig. 3). Binding of beads to the cell surface (determined by incubation of cells in the presence of sodium azide) was rapid and reached a maximum after 5 min of incubation, at approximately 15 beads 100 cells^{-1} (Fig. 3a). During the following 15 min, some release of beads from cells was evident, although subsequently, between 20 and 45 min of incubation, numbers of beads associated with the cell surface remained approximately constant at 7 beads 100 cells^{-1}. The proportion of cells associated with beads (either bound or phagocytosed) was also determined over the 45-min time course (Fig. 3b). Within 10 min of incubation in the presence of beads, approximately 80% of cells were associated with beads. During the subsequent 35 min of incubation, only a small further increase in the proportion of cells associated with beads was evident, indicating that those cells which were going to bind and/or phagocytose beads had largely done so within 10 min. After 45 min, approximately 92% of cells were associated with beads (Fig. 3b). Even after 2 h of incubation, when no further increases in total numbers of beads associated with cells were evident, a small fraction (<5%) of the cells displayed no fluorescence (results not shown).

The total number of beads associated with cells was calcu-

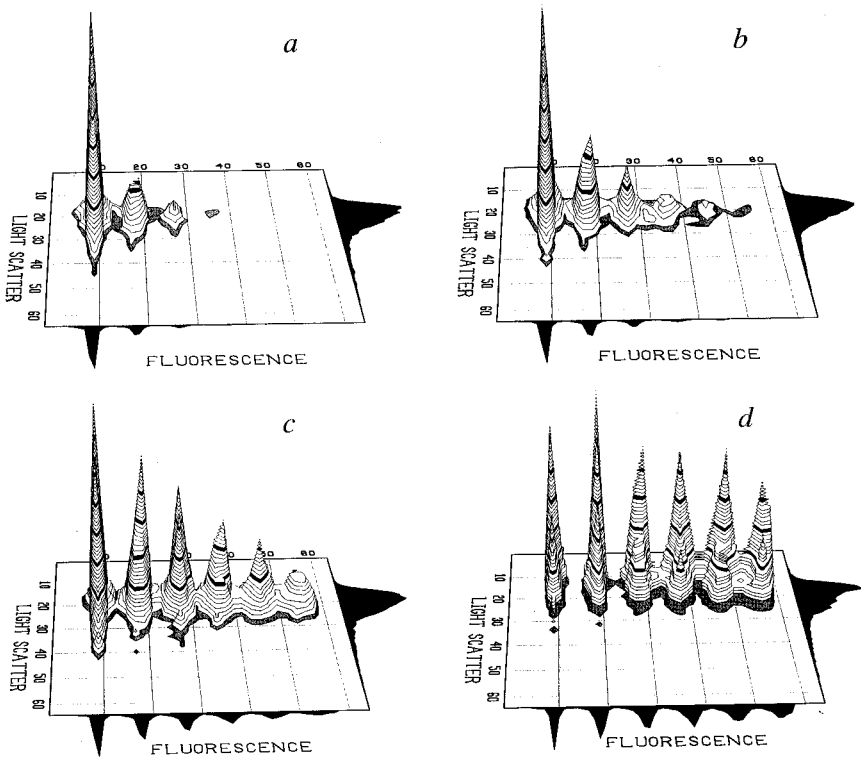

FIG. 4. Frequency distribution of cells with respect to forward-angle light scatter and fluorescence measurements. The corresponding single-parameter histograms are shown on the relevant axes. Fluorescence histograms (on a linear scale) show peaks corresponding to populations of cells that are associated with one to six (bound plus phagocytosed) beads. The figure shows distributions at 2 min (a), 5 min (b), 10 min (c), and 45 min (d) after the onset of phagocytosis (in cells incubated in the absence of sodium azide). Typical results from one of three experiments are shown. Each histogram contains data collected from at least 15,000 cells.

lated by determination of the total number of cells associated with each number of beads (i.e., by integration to determine areas under peaks in fluorescence histograms), multiplication of each pair of numbers, and addition of all the products. The total number of phagocytosed beads was determined by subtraction of the total number of bound beads from this figure. The total number of phagocytosed beads in *A. castellanii* cells increased approximately linearly over the first 30 min of incubation, at approximately 22 beads 100 cells^{-1} min^{-1}, although after 30 min a decline in the rate of bead accumulation was apparent (Fig. 3c). No further increase in the number of internalized beads was evident after 2 h (result not shown).

Isocontour plots of light scatter versus fluorescence at selected time points are presented in Fig. 4. For clarity, only peaks for cells with one to six beads (bound plus phagocytosed) are presented here. However, it should be noted that cells displayed a large degree of variability in their capacities to take up beads and some cells were found to contain >20 beads; resolution of fluorescence peaks was less clear at these high bead densities. After 2 min of incubation of cells in the presence of beads, the majority of cells displaying fluorescence had taken up only one bead and successively smaller numbers of cells were associated with peaks corresponding to two, three, and four beads (Fig. 4a). After 5 and 10 min of incubation, the relative proportion of cells containing more than one bead increased and peaks corresponding to cells containing five and six beads became evident (Fig. 4b and c). After 45 min of incubation, all fluorescence peaks were of similar heights (Fig. 4d). It should be noted that peak bases broadened with increasing fluorescence, and analysis of cell numbers associated with individual peaks revealed that more cells contained six beads than one bead after 45 min of incubation. Conversely, during incubation in the presence of sodium azide, cells associated with one bead always accounted for the largest proportion of fluorescently labelled cells (results not shown), as observed previously by Steinkamp et al. (49).

Numbers of cells containing one to six phagocytosed beads (calculated by subtraction of the equivalent figure for bound beads) were determined over the 45-min time course (Fig. 5). The proportion of all phagocytic cells that had internalized one bead increased rapidly to approximately 57% within 2 min of incubation (Fig. 5a). A subsequent decline in numbers of phagocytic cells containing one bead coincided with increases in the proportions of cells containing two to six phagocytosed beads. After 45 min of incubation, virtually no cells contained only one phagocytosed bead, whereas populations of cells containing two to six beads each accounted for approximately 8% of all phagocytic cells (where a population is defined as a group of cells containing a defined number of beads) (Fig. 5a). The precursor product pattern of numbers of beads phagocytosed in *A. castellanii* is clearer in Fig. 5b, in which individual population numbers are plotted as percentages of their maximal values during the time course. Cell populations containing increasing numbers of beads peaked at increasing lengths of time from the onset of phagocytosis. Thus, populations containing one, two, three, four, five, and six beads reached their maxima at approximately 2, 5, 10, 10, 15, and 20 min, respectively; after 45 min, cell numbers in each population were at approximately 0, 26, 43, 57, 73, and 81% of their maxima, respectively (Fig. 5b). Cell populations containing >6 phagocytosed beads reached their maxima after 20 min (results not shown).

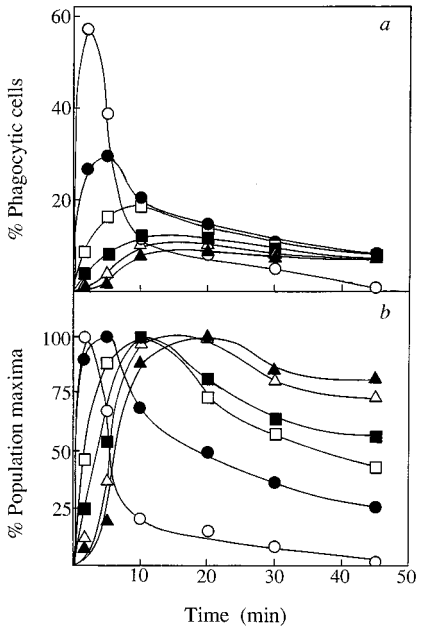

FIG. 5. Changes in numbers of cells in populations containing different numbers of phagocytosed beads. Numbers of cells containing one (○), two (●), three (□), four (■), five (△), and six (▲) phagocytosed latex beads were determined at specified intervals after the addition of beads to cell suspensions. (a) Percentages of phagocytic cells containing the specified number of phagocytosed beads. (b) Numbers of cells in populations containing the specified number of phagocytosed beads, expressed as percentages of maximum individual population numbers during the time course. Points are averages from three replicate samples, each representing data collected from at least 15,000 cells, ± standard errors of the mean where these values exceed the dimensions of the symbols.

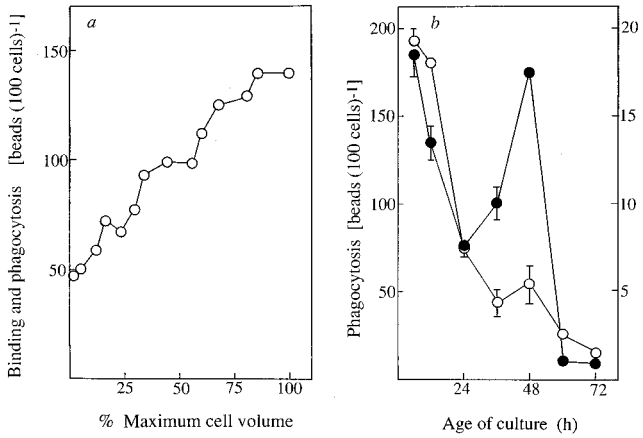

FIG. 6. Influence of cell volume (cell age) and culture age on phagocytosis in *A. castellanii*. Cells were incubated in the presence of latex beads for 10 min prior to analysis. (a) Total numbers of beads (bound plus phagocytosed) associated with fractions of cells of differing volume (see text). (b) Numbers of beads bound (○) and phagocytosed (●) in cells from cultures of differing ages. Points are averages from 2,000 (a) or 3 × 15,000 (b) cells, ± standard errors of the mean (error bars) where these values exceed the dimensions of the symbols.

Influence of cell volume (cell age) and culture age on phagocytosis in *A. castellanii*. Forward-angle light scatter histograms (Fig. 1a) correspond to cell volume distributions in heterogeneous cell suspensions (36). Cell volume increases along the horizontal axis of histograms. Thus, by appropriate adjustment of cursors (thresholds) on light scatter channels, phagocytosis data were collected for fractions of cells of differing volume. Subsequent determination of the proportions of cells falling below (smaller cell volume) or above (larger cell volume) the selected channels allowed estimation of the relative volume of cells in the selected fraction, i.e., as a percentage of maximum cell volume. Fractions were selected so as to represent only 1 to 1.5% of total cell numbers in the sample, thus minimizing variability attributable to differences in cell volume within the selected fraction. Accumulation of 1-μm diameter latex microbeads alone has a negligible effect on the cell volume of *A. castellanii* (53). Data presented in Fig. 6a represent total numbers of beads associated with cells after 10 min, i.e., bound plus phagocytosed beads. These values reflect rates of bead uptake over the 10-min incubation period at the particular stage of the cell cycle examined. It is emphasized that the values shown do not refer to cumulative levels of beads accumulated through the cell cycle. Precise calculation of numbers of phagocytosed beads was not possible because of difficulties encountered in selecting fractions of cells of identical volumes in successive samples. However, at no stage did the proportion of bound beads exceed 15% of the total number of beads taken up.

Bead uptake was observed to increase with cell volume (Fig. 6a). The average number of beads taken up after 10 min in the smallest-volume fractions (youngest cells) was low, at approximately 46 beads 100 cells^{-1}. Maximal bead uptake was observed in the largest (oldest) cells, which were considered to be on the verge of cell division, at around 140 beads 100 cells^{-1}. The latter value was approximately threefold greater than that evident in the smallest-volume fractions; however, the increase in bead uptake with cell volume was not completely linear and showed suggestions of a periodic pattern (Fig. 6a).

Numbers of beads being bound to the cell surface and being phagocytosed in *A. castellanii* were also markedly dependent on culture age (Fig. 6b). Cells harvested from early-exponential-phase cultures (6 to 12 h) bound and phagocytosed large numbers of beads. Numbers of phagocytosed beads in these cultures varied between 130 and 185 beads 100 cells^{-1} after 10 min of incubation. As cultures progressed to the mid-exponential-growth phase (24 h), cells displayed a progressive decline in their ability to bind and phagocytose latex particles. However, whereas surface binding of beads continued to decrease in cells from older cultures, late-exponential-phase cells (36 to 48 h) displayed increased phagocytotic activity. Subsequent entry into stationary phase coincided with a very rapid and marked decline in the rate of phagocytosis, to approximately 5% of that observed at 48 h (Fig. 6b).

Influence of bead and cell density on phagocytosis in *A. castellanii*. Numbers of cell surface-bound and phagocytosed latex beads were determined at various bead densities, with the cell density maintained at approximately 10^7 ml^{-1} (Fig. 7a). Numbers of beads bound to the cell surface after 10 min increased linearly with the concentration of beads (Fig. 7a). A linear increase in numbers of phagocytosed beads was also evident at bead densities of up to approximately 1.5×10^8 ml^{-1}. However, further increases in particle concentrations, up to 2×10^9 beads ml^{-1}, caused no further increases in numbers of internalized beads, despite continued increases in numbers of bound beads (Fig. 7a).

A similar pattern was evident when the cell density was varied between 5×10^5 and 2×10^8 cells ml^{-1}, with that of latex beads maintained at 5×10^7 ml^{-1} (Fig. 7b). On a per-cell basis, numbers of beads bound to the cell surface decreased linearly with increasing cell density. In contrast, changes in numbers of phagocytosed beads at various cell densities were not always linear, and saturation of phagocytosis was evident at cell densities of $\leq(5 \times 10^6)$ cells ml^{-1}. Interestingly, assuming

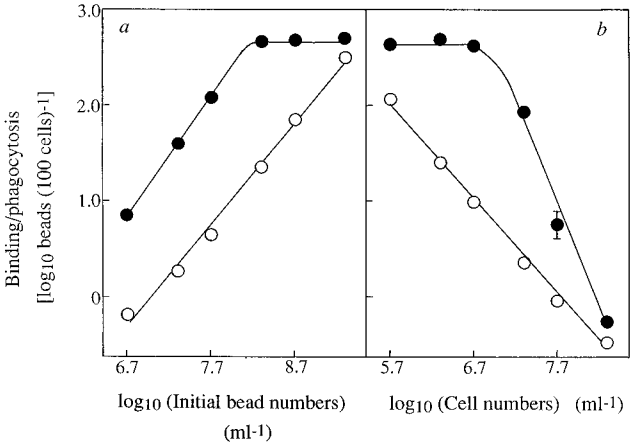

FIG. 7. Effects of bead and cell density on phagocytosis in *A. castellanii*. Cells were incubated in the presence of latex beads for 10 min prior to analysis. (a) Bead uptake at various initial bead densities and a cell density of approximately 10^7 ml^{-1}. (b) Bead uptake at various cell densities and an initial bead density of approximately 5×10^7 ml^{-1}. The graphs show total numbers of bound (○) and phagocytosed (●) beads. Points are averages from three replicate samples, each representing data collected from at least 15,000 cells, ± standard errors of the mean (error bar) where these values exceed the dimensions of the symbols.

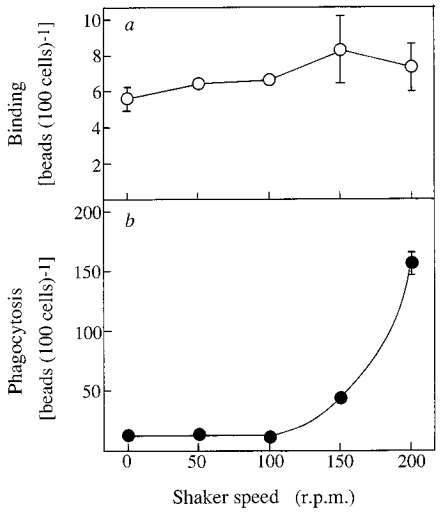

FIG. 8. Influence of rate of rotary shaking on phagocytosis in *A. castellanii*. Cells were incubated in the presence of latex beads for 10 min prior to analysis. Bead uptake was determined in cells incubated at the specified shaker speeds. (a) Total numbers of bound beads (○); (b) total numbers of phagocytosed beads (●). Points are averages from three replicate samples, each representing data collected from at least 15,000 cells, ± standard errors of the mean (error bars) where these values exceed the dimensions of the symbols.

that numbers of phagocytosed beads increased approximately linearly with time over the first 10 min of incubation in all cases, the calculated rates of phagocytosis at apparent saturation in both Fig. 7a and b were similar, at approximately 0.5 beads cell^{-1} min^{-1}. The bead-to-cell ratios at which phagocytosis became saturated in the two experiments were of the same order; approximate values were 15:1 and 10:1 at increasing bead and decreasing cell densities, respectively.

Influence of rate of rotary shaking on phagocytosis in *A. castellanii*. Binding and phagocytosis of latex beads were examined in cells incubated at various rates of rotary shaking. Increasing the shaker speed from 0 to 200 rpm resulted only in an increase in the average number of beads bound at the cell surface from approximately 6 to 7 beads 100 cells^{-1}, after 10 min of incubation (Fig. 8a). In contrast, shaker speed had a very pronounced effect on phagocytosis. At shaker speeds between 0 and 100 rpm, average numbers of phagocytosed beads were low, at approximately 10 beads 100 cells^{-1} after 10 min (Fig. 8b). However, phagocytosis was markedly stimulated at rates of rotation exceeding 100 rpm, and the average number of beads phagocytosed at 200 rpm was approximately 16-fold higher than that at 100 rpm (Fig. 8b). Cells began to lyse at shaker speeds exceeding 200 rpm.

DISCUSSION

This paper shows that the use of flow cytometry has many advantages over more conventional techniques for the measurement of phagocytosis in protozoa. The most important benefit is that very large numbers of cells can be rapidly analyzed, e.g., approximately 15,000 cells in 1.5 min in the present study; an equivalent feat by microscopy would require several hours of patient counting. Furthermore, flow cytometry requires only small sample sizes and differentiates between individual cells according to the number of particles (if fluorescently labelled) each has accumulated. This is particularly important considering the high degree of variability between individual cells observed here; resolution of cellular heterogeneity would not be possible by spectrophotometric methods or supernatant counts, as these give only an average value for phagocytosed beads per cell.

In the present study, fluorescently labelled latex microbeads were used as a substrate for phagocytosis in *A. castellanii*. High degrees of homogeneity with respect to particle size and fluorescence intensity are prerequisites for flow-cytometric investigations of the nature described here. Such homogeneity is typical of commercially available latex beads but is very difficult to establish by labelling of more natural (so-called) phagocytic substrates, e.g., bacterial and yeast cultures. Latex particles have been used extensively in phagocytosis studies (11, 18, 48, 54). Latex beads differ in surface properties from natural substrates such as yeasts or bacteria, in which uptake is mediated by mannose receptors at the amoeba surface (3). However, whereas *A. castellanii* and other protozoa still display very effective discrimination in the uptake of different microbial species or even strains (10, 40, 52), discrimination against latex beads is more marked at the level of digestion than at that of internalization (12, 41). Thus, the use of uniform latex beads for phagocytosis research is advantageous for a number of reasons. For example, they allow estimation of the surface area of internalized membranes (11, 48), facilitate the isolation of phagocytic vesicles (50), and permit the study of those cellular changes that are entirely attributable to internalization (rather than digestion) (53). Furthermore, because of its chemically inert nature, interactions involving latex are far simpler to analyze than those involving incorporation of microbial biomass and/or organic matter (53).

Separate determination of binding and internalization processes was achieved by incubation of cells in the presence or absence of sodium azide. The rapid saturation of cell binding sites followed by a partial detachment of bound particles reported here is also known to occur during binding of heat-killed yeast cells to *A. castellanii* (2). Flow-cytometric and microscopic analyses indicated that no loss of cellular integrity resulted from incubation in the presence of sodium azide, and the observed decline in bound particles suggests the possible action of a release mechanism for nonphagocytosed or egested

particles. The proportion of cells that were associated with latex beads in phagocytosing cultures also increased rapidly. In contrast, increases in the total number of beads phagocytosed by *A. castellanii* were more gradual and, as shown in previous studies (44, 48, 53, 54), phagocytosis was approximately linear over the first 30 min of incubation. These differences were accounted for by a time-dependent increase in the number of beads internalized in individual cells. Thus, most cells became rapidly associated with one bead within a few minutes and populations containing increasing numbers of beads peaked at increasing intervals of time. Results presented by Weisman and Korn (53) hinted at a similar pattern of time-dependent bead uptake in *A. castellanii*; however, these workers counted only 100 cells per sample microscopically, thus precluding the detailed analysis presented here. The decline in the apparent rate of phagocytosis after 30 min that is reported here is probably not the result of a genuine decrease in the rate of bead internalization. A similar levelling off of numbers of internalized bacteria in marine protozoa was attributed by Sherr et al. (46) to an equilibration of rates of uptake and digestion. However, the indigestible nature of phagocytosed latex renders this explanation implausible here; a more likely explanation is that a plateau in the numbers of internalized beads is reached when rates of uptake are matched by rates of egestion. Phagosomes carrying indigestible material in *A. castellanii* (12) and other protozoa (40) have been observed to migrate towards the cell surface, where the contents are eventually expelled, after a period of time. As the rate of accumulation of beads declined after 30 min of incubation in the present study, this incubation period probably represents the minimum time required for cells to complete the processes of ingestion and egestion of a single particle under the conditions described. Similarly, the maximum retention time of a phagocytosed bead can be estimated to be approximately 2 h, as internalization and egestion are in apparent equilibrium after this time.

In the present study, appropriate setting of thresholds on forward-angle light scatter channels during data collection allowed phagocytosis to be analyzed in cells differentiated by volume. Different fractions actually represented cell populations at varying stages of the cell cycle, as the cell volume of *A. castellanii* increases through the cell cycle (13, 14). Thus, a cell will attain maximum volume just prior to division and the average cell volume will be smallest in newly divided cells. This method of cell cycle analysis is entirely nonperturbing and circumvents many problems associated with the production of synchronous cultures for such studies, e.g., perturbation of metabolism during synchrony induction (35). Thus, the technique is unique in allowing differential cell cycle analysis in large numbers of cells from cultures displaying cell cycle heterogeneity. The rate of phagocytosis in *A. castellanii* increased with cell volume; this increase may be attributable to the larger plasma membrane surface area and increased nutritional requirements of larger cells. Thus, older cells had a greater capacity to ingest particles from their external medium than young cells, although at no stage in the cell cycle did phagocytosis cease completely. A similar cell cycle dependence of phagocytosis has recently been reported for *Tetrahymena pyriformis* (27). However, elsewhere it has also been demonstrated that phagocytosis ceases between 5 and 35 min after cell separation in *Tetrahymena* spp. (40). The present study indicates that, in contrast to previous speculation (12), the small but consistent population of nonphagocytosing cells observed here, and elsewhere (12), cannot be accounted for by a nonphagocytic stage of the *A. castellanii* cell cycle. However, it should be noted that increases in phagocytosis with cell volume showed indications of periodicity, and the possible operation of an ultradian rhythm (36) of phagocytosis cannot be discounted.

Marked changes in phagocytotic activity were also evident in cells at different stages of growth in batch culture and, in agreement with previous studies (11, 44), rates of phagocytosis were very low in stationary-phase cultures. High rates of phagocytosis in early-exponential-phase cultures appeared to be a direct result of enhanced binding of beads to the cell surface. However, a rise in phagocytosis in late-exponential-phase cultures was contemporaneous with a continued decline in the apparent affinity of beads for the cell surface. Thus, phagocytosis in *A. castellanii* appears to be controlled by factors which govern the rate of membrane invagination and particle internalization as well as by the degree of cell surface particle binding. Factors which may contribute to these fluctuations include changes in plasma membrane properties, e.g., fluidity (4, 5, 11), nutrient deprivation (40), the presence of particles produced during autoclaving of media (23), and controls exerted by growth-dependent changes in the capacity of the digestive system of *A. castellanii* (20).

As with growth-dependent changes in phagocytosis, changes in numbers of internalized beads at increasing cell or bead densities were not strictly related to changes in the numbers of beads being bound. Increases in rates of particle uptake (on a per-cell basis) with concentrations of particles and with decreases in cell concentration of *A. castellanii* are well documented (23, 53). However, in this study the phagocytotic apparatus of *A. castellanii* eventually became saturated, while binding of beads at the cell surface continued to increase. These results therefore confirm several earlier suggestions that the phagocytotic capacity of *A. castellanii* is defined by the intracellular vacuolar system of the organism (11, 12, 43) and not by the number of available binding sites at the cell surface (53). (It should be noted that a further limitation may be imposed on digestible material by the rate of intracellular hydrolase compartmentation [30].) Interestingly, apparent saturation of phagocytosis occurred at bead-to-cell ratios of approximately ten or fifteen to one in the present study. This is far lower than the relative numbers of particles and cells employed in many other studies, in which ratios ranging from 40:1 (3) to 2,000:1 (54) are commonplace. It is likely that the latter ratios are far in excess of those required to saturate the phagocytotic apparatus of *A. castellanii*, and in these cases the effects of independent experimental parameters on phagocytosis may be masked.

The observed enhancement of phagocytosis at high speeds of rotary shaking was unexpected. First, these changes were not attributable to increased binding between cells and beads, as evidenced from data collected for cells incubated in the presence of sodium azide. Second, settling and attachment of nonagitated amoeboid cells to solid surfaces are facilitated by the formation of pseudopodia; under these circumstances, an increased tendency to phagocytose surrounding particles might be expected. Indeed, several previous studies have examined phagocytosis in protozoa and in mammalian cells when the cells are attached to slides or other solid surfaces (28, 38, 45, 51). It seems likely that maximal rates of particle ingestion may not have been attained under these conditions. As phagocytosis in *A. castellanii* is energy dependent (3, 53), it is possible that the present observations may be attributable to enhanced rates of oxidative metabolism at increased dissolved oxygen concentrations in agitated cultures.

Thus, separate analyses of the processes of bead binding and internalization throughout the present study have yielded further clues relating to factors controlling phagocytotic activity under different conditions in *A. castellanii*. The present flow-

cytometric method may also be used in conjunction with fluorescence quenching techniques (28, 29, 38) or with confocal scanning microscopy (31) to provide rapid information on numbers of internalized beads or on intracellular bead localization, respectively. The results presented here emphasize the marked dispersion of phagocytotic rates both between individual cells and between whole populations of *A. castellanii* under different conditions. These considerations may have important implications for protozoal grazing in the natural environment and its potential impact on nutrient recycling and remineralization. The ability to rapidly analyze large numbers of cells and to fractionate populations by cell cycle stage or by the number of particles phagocytosed in individual cells renders flow cytometry superior to and more powerful than earlier methods for studies of protozoal phagocytosis.

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