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Computer Image Analysis Method for Rapid Quantitation of Macrophage Phagocytosis

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A new method is described for rapidly quantitating phagocytosis by adherent macrophages in culture using computer image analysis (CIA) of video light microscopic images. Ingestion of fluorescent microspheres by peritoneal murine macrophages is used to model phagocytosis. The grey levels of digital phase contrast and fluorescent microscopic images are used to quantitate the number of microspheres per cell. The method is semi-automatic, analyzes approximately 2 × 10^3 cells/hr, and simultaneously measures phagocytosis (microspheres/cell), cell area, and density (number of cells/mm^2). CIA obtains the same microspheres/cell average as manual microscopic counting and an analytical precision of 5%. As expected, CIA found that the number of microspheres/cell linearly increases with increasing macrophage-microsphere co-culture time or increasing microsphere concentration until macrophages become saturated. CIA finds increased phagocytosis by interferon-gamma-treated cells and suppressed phagocytosis by cytochalasin B- or 4°C-treated cells relative to controls, which demonstrates that CIA can resolve biological changes in macrophage phagocytosis. CIA also provides quantitative data on macrophage morphometry and density and found an increase in the cell area and density of INF treated macrophages. CIA provides significantly more phagocytic, morphometric, and density data than conventional manual microscopic counting methods or flow cytometric methods. The limitations, improvements, and future applications of this method are discussed.

Key words: computer image analysis, macrophages, phagocytosis, drug evaluation

INTRODUCTION

Macrophage phagocytosis has long been recognized as a critical component to the immune response against bacterial infections [24]. Defective phagocytic function has been associated with increased susceptibility to infection [6,16,19]. Recombinant cytokines show promise as therapeutic agents for combating bacterial infections, and may act by enhancing phagocytosis [8,27,33,34]. Macrophages are also exceedingly pleomorphic cells and their structure depends on the state of activation. Yet, quantitating macrophage phagocytosis and structure in response to cytokines has been limited by available analytical methods.

Current single cell methods for quantitating phagocytosis are either direct microscopic examination [10] or by flow cytometric analysis [1,5,31]. Light microscopic examination and manual counting of particles in individual cells is the most common method of quantitating phagocytosis, but only a few cells can be analyzed and no quantitative morphometric data are obtained. Flow cytometry can quantitate phagocytosis of many cells in suspension [11,26], but cannot provide detailed morphometric data. Computer image analysis of microscopic images can provide detailed quantitative morphometric data [3,4,9,12,14,15] of adherent cells, but image analysis methods have not been developed to quantitate phagocytosis.

Presented here is a method of image analysis that quantitates phagocytosis and morphometry of adherent macrophages observed with a light microscope. This method offers significantly faster phagocytic analysis than manual microscopic examination and more detailed quantitative morphological data than flow cytometry. In this report, the technical details of the analysis procedure and tests of its accuracy and reproducibility are described.

MATERIALS AND METHODS

Equipment

The light microscope and computer image analysis system are diagramed in Figure 1. The computer image

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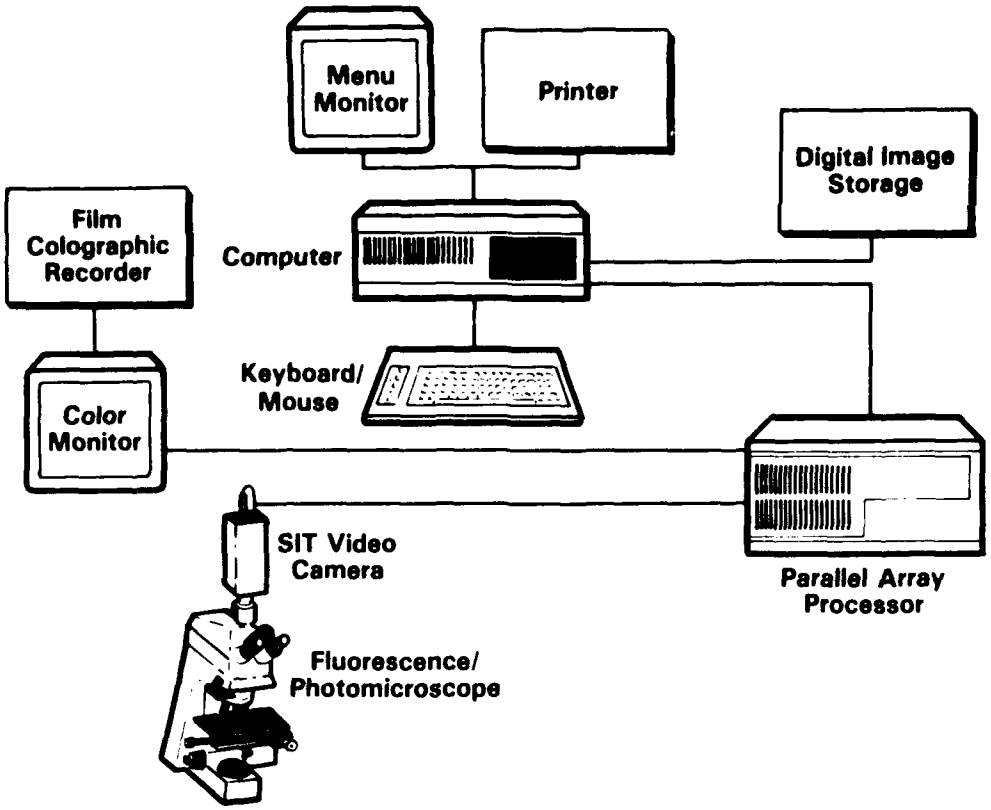

Fig. 1. A diagram of the image processing system used for macrophage phagocytosis is shown. A SIT video camera is used as the image forming system for on-line analysis.

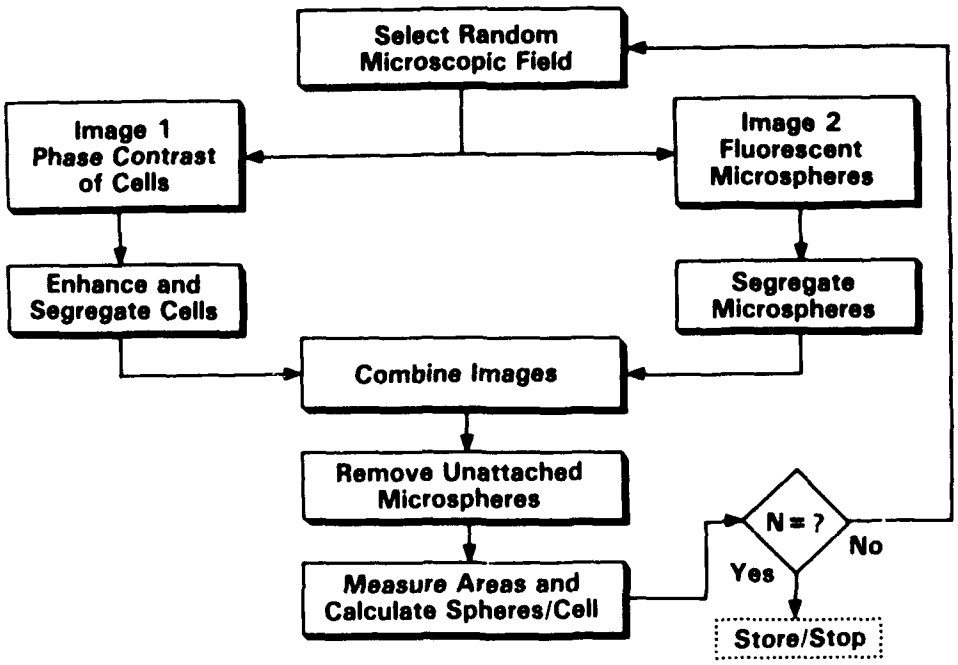

Fig. 2. Schematic flow diagram of the image analysis procedure. The user selects random microscopic fields from which phase-contrast and corresponding fluorescent images are taken separately, segregated, and added to measure cell areas and calculate the number of microspheres per cell.

analysis system is a Kontron-Zeiss SEM-IPS (Carl Zeiss, Inc., Thornwood, NY) attached to an upright light microscope (Photomicroscope II, Carl Zeiss, Inc., Thornwood, NY) equipped with phase-contrast optics, mercury arc epi-illumination (50 watts), and fluorescence bandpass filters (458 nm excitation, 540 nm emission center wavelength). A low light silicon intensified target video camera (SIT 66, DAGE-MTI Inc., Michigan City, IN) made video images of the cells and microspheres. The on-line video images were digitized via a parallel array processor under interactive control of a Zilog Z80A (4 MHz)-based host computer with CP/M operating system. Digitized images were stored in one of the seven 512 × 512 × 8 bit volatile memory planes of the SEM-IPS during analysis. The number of memory planes is limited by the number of memory chips installed. The images can be stored permanently on 10 megabyte removable IOMEGA disks.

A commercially available software system, IBAS, release 4.4 (Carl Zeiss, Inc., Thornwood, NY) was used to analyze the images. The IBAS, a menu driven system, has a large selection of image acquisition, enhancement, analysis, and display subroutines. The IBAS may be used to create a macro program consisting of both IBAS and user-written subroutines that may be automatically executed with IBAS' subroutines if the system is so instructed. Although this program will run only within the IBAS environment, the basic principle of the method can be adapted and used on other image analysis systems.

Computational Methods

The schematic flow diagram of the CIA procedure is shown in Figure 2. Non-overlapping random fields of cells were examined using a phase-contrast microscope with a 25×, NA 0.45, objective lens. This magnification maximized the number of cells observed while providing sufficient magnification and resolution of the microspheres and cells. After optimal adjustment of the video camera, phase-contrast and epi-fluorescence video microscopic images of each field were sequentially recorded. A video phase-contrast image of the cells was obtained after adjusting the light and video camera to maximize the contrast. The video image was digitized, frame-averaged (three times), and stored in a computer image plane (random access memory). Without changing the field, a fluorescent image of the microspheres was made by turning off the phase-contrast illumination and turning on the epi-fluorescent illumination. The fluorescent image was digitized, frame averaged, and stored in a separate image plane.

To remove artifacts in the image caused by dirt in the optical system, a defocused background image frame was subtracted from the original image frame. This was done by defocusing the microscope, frame-averaging, digitizing, and storing the background phase-contrast image frame. To make the cells completely dark, the resulting image was scaled by making grey values from 0 to 120 black (0) and 121 to 255 white (255), then enhanced by a ranked median filter (kernel size 5 × 5, rank 13). The ranked median filter is a group-like process operating on a 5 × 5 pixel neighborhood. The center-pixel grey value output of the filter is the median value of the 25 pixel brightness values. The median value of a neighborhood is determined by placing the 25 brightness values into ascending numerical order and selecting the center value to replace the original grey value.

The cells within the enhanced phase-contrast field were selected by grey level discrimination and transformed into a binary image. The first field discrimination was done interactively by the operator; the rest of the fields were discriminated automatically using the selected grey value window to produce consistent grey value-dependent object areas. The fidelity of the discrimination was evaluated by overlaying contour maps of the binary images on the original phase-contrast images. The fluorescent microsphere images were also discriminated from the background and inverted to black so that the microspheres could be differentiated from the cell. The cell and fluorescent binary images were added and stored. The cells and microspheres have grey values of 255 (white) and 0 (black), respectively, in the composite image (see Fig. 3). The number of microspheres in each cell was calculated from the composite binary image. Microspheres not associated with cells were eliminated from the analysis by size "thresholding."

The number of microspheres per cell was calculated by dividing the total microsphere area in each cell by the area of a single microsphere. From the areas of the black microspheres and white cells, the mean grey value (GV) was computed and is proportional to the cell area occupied by microspheres. The GV, which is the weighted average of the microsphere area (M) and the cell area (C), is given by

$$GV = ((0 * M) + (255 * C))/AR,$$

where AR is the total area of both cells and microspheres. The proportion of each cell's area occupied by microspheres (A) is

$$A = (1 - (GV/255)),$$

and total microsphere area of each cell (MA) is

$$MA = A * AR.$$

The number of microspheres per cell (MC) is

$$MC = MA/SM,$$

where SM is the area of a single microsphere. Since only whole microspheres are possible, the quotient is rounded off to the nearest whole integer.

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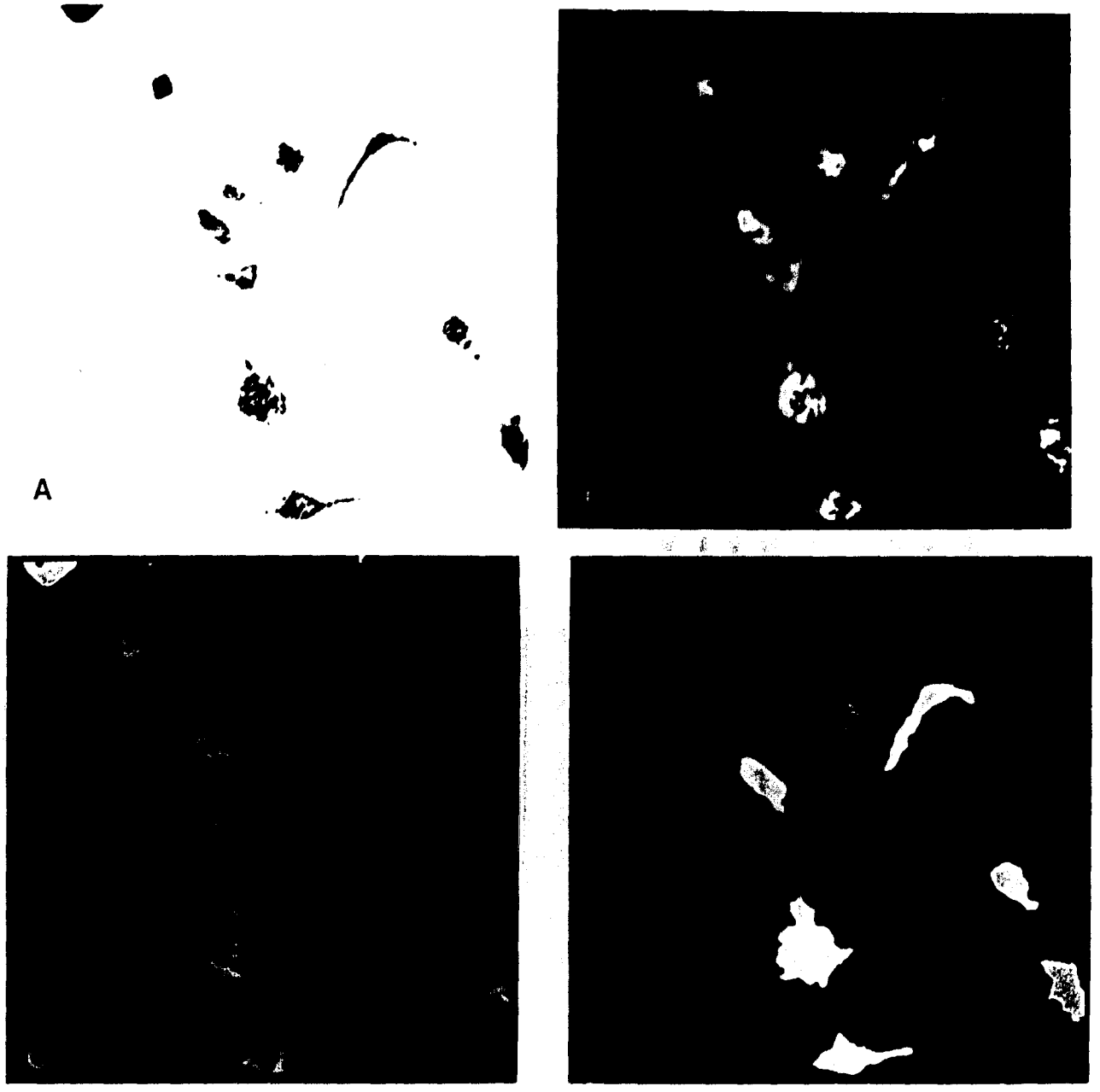

Fig. 3. The sequence for measuring number of microspheres per cell using CIA. A: Original video-digitized image of cells (×25, NA 0.45). B: Corresponding original fluorescent image of microspheres from the same field as A. C: Binary cell (light blue) and fluorescent microsphere (dark blue) images added together. D: Processed field from which background microspheres have been eliminated before measurement; random colors assigned and signify each cell individually analyzed.

Macrophage Harvest and Phagocytosis

Standard peritoneal lavage and culture techniques were used to obtain murine peritoneal exudate cells and macrophage-enriched cell cultures [22]. Ten- to 12-week-old female BALB/CJ mice (Jackson Laboratories, Bar Harbor, ME) were sacrificed by cervical dislocation by an individual trained in such euthanasia. Animals were placed in a supine position, the abdominal area was wetted with 70% alcohol, and the abdominal skin was cut and retracted. The animals were injected intraperitoneally with 4 ml cold Hanks' Balanced Salt Solution (HBSS, GIBCO Laboratories, Grand Island, New York), gently rotated and shaken (1 min), incised below the sternum, and peritoneal exudate fluid was removed using

a sterile Pasteur pipet. The exudate cells were pooled into 50 ml centrifuge tubes (Labcon, San Rafael, CA) and washed twice in cold HBSS by centrifugation at 200*g* for 10 min at 5°C (CRU-50, Damon/IEC Division, Needham Hts, MA). The pellet was reconstituted in RPMI 1640 growth media (Flow Laboratories, McLean, VA) containing 10% fetal calf serum (FCS, lot #27N1072, Gibco Laboratories, Grand Island, NY), 2% HEPES, 0.1% gentamicin, and 1% glutamine to a concentration of approximately 2.5×10^6 cells/ml. Peritoneal exudate cells, plated at 2.5×10^6 cells/dish in 3 ml of media in tissue culture dishes (60 × 15 mm, Costar, Cambridge, MA), were incubated in a 5% CO_2 atmosphere at 37°C overnight (Model 3185, Forma Scientific, Marieta, OH). Non-adherent cells were removed from the cultures by briskly rinsing each dish three times with 3 ml/wash of cold HBSS.

To determine if CIA could resolve biological changes in macrophage phagocytic function, marcrophages were cultured with agents known to affect phagocytosis. To inhibit phagocytosis, microspheres and macrophages were either co-cultured at 4°C or macrophages were pretreated with cytochalasin B (4×10^{-5} M, Sigma, St. Louis, MO) for 1 hr before and during microsphere co-culture. To enhance phagocytosis, macrophages were cultured with recombinant murine interferon-gamma (INF, Genzyme, Boston, MA) at various concentrations (10, 100, and 1,000 U/ml) 24 hr before microsphere co-culture. As a control for possible lipopolysaccharide (LPS) contamination of INF, an aliquot of INF (1,000 U/ml), was heated (100°C, 1 hr) to denature it (but not LPS) before adding the INF to the cultures.

Macrophage culture media was decanted off and fluorescent microspheres of various known concentrations in growth media were added to the cultures (3 ml/dish). Concentrations of 2×10^8 and 4×10^8 microspheres/ml were used to give cell-to-microspheres ratios of 1:80 and 1:160, respectively. The macrophage and microsphere co-cultures were incubated in 5% CO_2 at 37°C for various lengths of time. After incubation, the cultures were jet-stream washed (3×, cold HBSS), fixed (10 min, cold 2% glutaraldehyde, 2 mM $CaCl_2$ in 0.1 M cacodylate buffer pH 7.2), washed (3×, 0.1 M cacodylate buffer), stained (2 min, Wright-Giemsa quick stain, Curtin Matheson Scientific, Inc., Houston, TX), decanted, and dried before analysis. The staining procedure was repeated if cells were not dark enough for segregation.

Microspheres

Uniformly sized fluorescent carboxylate microspheres (1 μm diameter 1.5% standard deviation, yellow-green fluorescence, 458 nm maximum excitation, 540 nm maximum emission, Catalog #15702, Polysciences, Warrington, PA) were used for all studies. A stock microsphere suspension (200 μl) was washed in HBSS by centrifugation (11,950*g*, 15 min, 5°C, Sorvall RC-5B, DuPont Instruments, Newtown, CT) and resuspended in growth media (40 ml). To determine the concentration of microspheres, an aliquot of the suspension was diluted in distilled water and analyzed by CIA. Three drops (1 μl each) of the appropriate dilution were placed on a clean glass microscope slide, dried, and counted using the microscope and CIA system. Microspheres in the entire area of the drop were counted and the number of microspheres per ml was calculated from the result based on the dilution. The theoretical microsphere concentration (TC) was calculated and compared to CIA analysis using the following:

$$TC = (6W * 10^{12})/(p * \pi * d^3),$$

where W is the concentration of polymer (0.025 g/ml), p is density of polymer (1.05 g/ml), and d is the diameter of the microspheres (1 μm). Accuracy and precision was improved by calibrating the number of microspheres in a field obtained by CIA to the number obtained by manual methods before beginning analysis of an unknown.

TABLE 1. A Comparison Between the Mean Number of Microspheres Per Cell Determined by CIA and Manual Microscopic Counting[a]

No. field	No. of cells	Manual count (spheres/cell)	CIA (spheres/cell)	P*
6	69	17.3 ± 11.4	16.3 ± 10.9	0.144

[a]Values are given as mean ± SD. The cells and microspheres were co-cultured for 1 hr at a cell-to-microsphere ratio of 1:160.
*No significant difference by paired Student's t test.

RESULTS AND DISCUSSION

No significant difference was found in the mean number of microspheres per cell determined by CIA and manual microscopic counting ($P = .144$, Student's t test, N = 69, see Table 1). The results of repetitive analyses of one field by three operators are shown in Table 2. The difference in CIA-determined counts between and within operators is not significant ($P = .94$, one-way analysis of variance, N = 24). Figure 4 shows that the number of microspheres per cell linearly increased with increasing microsphere-cell co-culture time ($r^2 = 0.98$) and the number of microspheres per cell was greater for higher microsphere-to-cell ratios at a given co-culture time. Phagocytosis was completely suppressed by co-culturing the microspheres and cells at 4°C. We conclude that CIA can produce accurate data on phagocytosis with reasonable precision.

CIA may be particularly useful for determining macrophage sub-classes based on phagocytosis. Figure 5 is a

TABLE 2. Variability Among and Within Operators Using CIA to Analyze One Field Three Times[a]

Operators	Analyses 1	2	3	CV (%)[b]
1	14.0 ± 12	13.8 ± 12	14.0 ± 12	.6
2	15.0 ± 11	15.1 ± 13	13.4 ± 11	5.0
3	16.5 ± 14	13.0 ± 11	14.3 ± 12	10.0
CV[c]	6.7%	6.0%	2.6%	

[a]Values are given as mean ± SD, N = 8 cells.
[b]Coefficient of variation among analyses for a given operator. Operator 1 was trained whereas operators 2 and 3 were naive. The grand mean CV among analyses equals 5.2%.
[c]Coefficient of variation among operators. The grand mean CV among operators equals 5.1%.

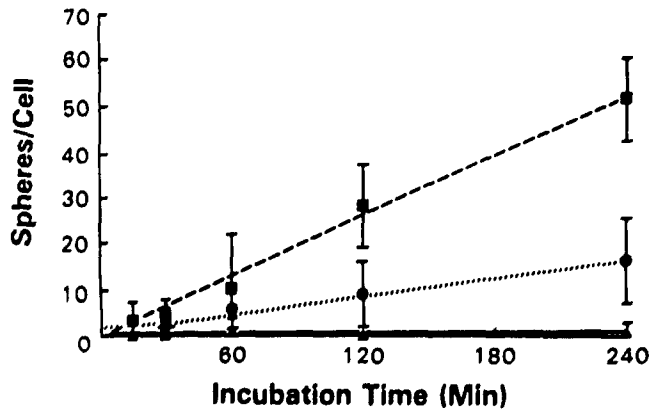

Fig. 4. A comparison of the time-course of phagocytosis with respect to increase in the concentration of microspheres and incubation temperature. With increasing incubation time and 37°C, the number of microspheres per cell increased for 1:80 (. . .) and 1:160 (- - - -) cell-to-microsphere ratios. No phagocytosis occurred for the assay performed at 4°C. The display represents the mean ± one standard deviation, N = 1,500 cells/point.

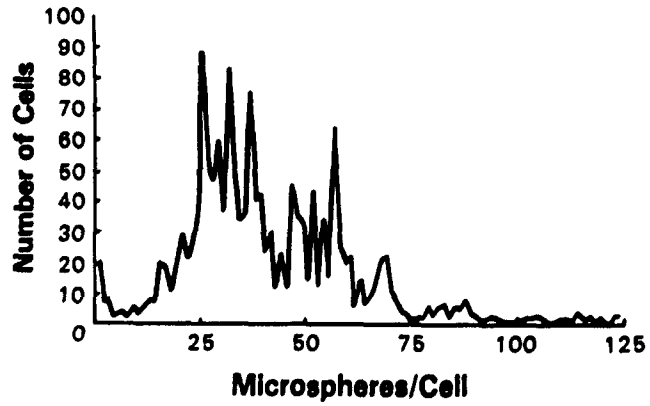

Fig. 5. A typical histogram of microspheres per cell indicating possible bimodal distribution with peaks centered around 25 and 55 microspheres per cell. The cells and microspheres were co-cultured for 2 hr at a cell-to-microsphere ratio of 1:160.

typical histogram of the number of microspheres per cell from analysis of 1,500 cells obtained in approximately 45 min. The histogram suggests there is a possible bimodal distribution in the number of microspheres per cell in the population. Because of the inherent functional heterogeneity reported for phagocytic cells [7,13,20,32], analysis of a large number of cells is essential to resolve macrophage sub-classes. Further, CIA has the potential to measure three other phagocytic parameters that should allow more detailed analyses of macrophage sub-classes. From the number of microspheres per cell and the number of cells analyzed, other important phagocytic parameters [28,30,31], the percent phagocytic cells (phagocytic activity index), the number of microspheres phagocytized per phagocytic cell (avidity index), and the total number of microspheres phagocytized (phagocytic capacity), can be calculated. These additional parameters could be important for determining macrophage subclasses and will be incorporated into future versions of the algorithm.

A major application of CIA will be screening for the effects of drugs on macrophage phagocytosis. Figure 6 shows the stimulatory or inhibitory effects of INF or cytochalasin B, respectively, on phagocytosis. The number of microspheres per cell for INF-treated cells was significantly greater than control cells ($P < .01$, Student's t test, N = 1,500 cells/group). IFN is a known macrophage activator [21,23,25]. Significant suppression of phagocytic function was found for cytochalasin B, a known phagocytic function inhibitor [2] ($P < .01$, Student's t test, N = 1,500 cells/group). Previously, CIA found enhanced macrophage phagocytosis of interleukin-4 (IL-4)-treated cultures, which was dose and time dependent [18]. The IL-4 enhancement results found by CIA are in agreement with those reported using conventional phagocytosis methods [34]. Therefore, CIA will be useful for screening the effects of chemicals such as cytokines or bacterial virulence factors on phagocytosis, the development of anti-microbial agents, and in pathogenic model systems.

In addition to analyses of phagocytosis, CIA provides morphometric and cell density measurements. As shown in Figure 7, INF had a pronounced effect on macrophage cell area and density. Other morphometric parameters, such as perimeter and shape factor (perimeter^2/area), can be obtained for more detailed analyses. Morphometric and cell density measurements will be used to analyze the effects of cytokines on macrophage structure-function relations.

To achieve precise and consistent results, uniformly stained cells and relatively few non-phagocytozed (background) microspheres were required. The sample preparation described routinely achieved these critical condi-

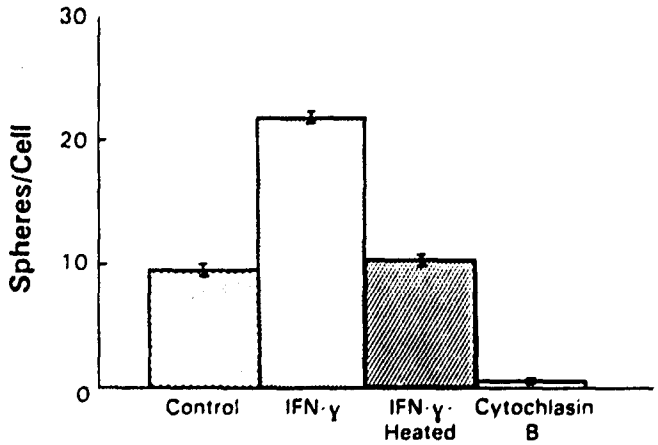

Fig. 6. Phagocytic response of macrophages to heated and unheated INF, and cytochalasin B. IFN increased phagocytosis and cytochalasin B decreased phagocytosis relative to controls ($P < .01$, Student's t test, N = 1,500 cells/group). Heated gamma-interferon had no effect relative to control. The display represents the mean ± one standard error of the mean, N = 1,500 for each point.

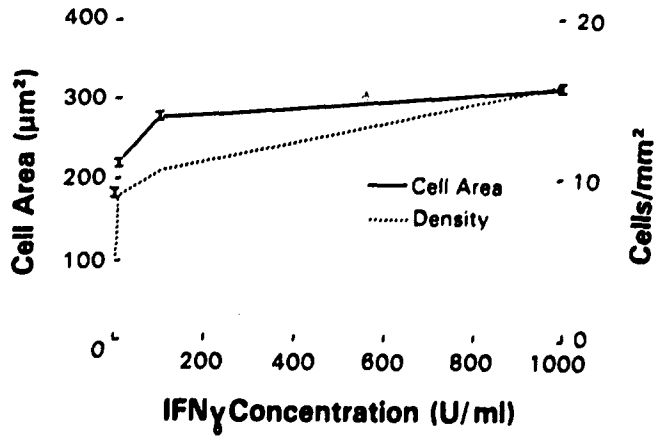

Fig. 7. Morphometric response of macrophages to increasing concentration of INF showed that cell area (——) and density (- - - -) increased with INF concentration. Increased cell area is due to activation by INF. The display represents the mean ± one standard error of the mean, N = 1,500 for each point.

tions. Non-clustered, well stained cells and few non-phagocytosed microspheres, made automatic segregation easy and fast (approximately 2×10^3 cells/hr).

Although CIA has many advantages, there are also several inherent limitations. For example, the CIA measured concentration of the microspheres/ml (Fig. 8) showed a linear relationship ($r^2 = 0.98$) up to 1×10^8 microspheres/ml, but significantly underestimated higher concentrations. The reason for this is that many microspheres overlay each other in the higher concentrations and CIA determines the number of microspheres based on the projected area, which means that overlaying microspheres are counted as one microsphere. This is not a significant problem for macrophage phagocytosis measurements if the microspheres per cell are not excessively large. As shown in Figure 4, the linear response between microspheres per cell and macrophage-microsphere coculture time indicates that under the conditions used, microsphere pile-up in macrophages did not have a significant effect on the analysis.

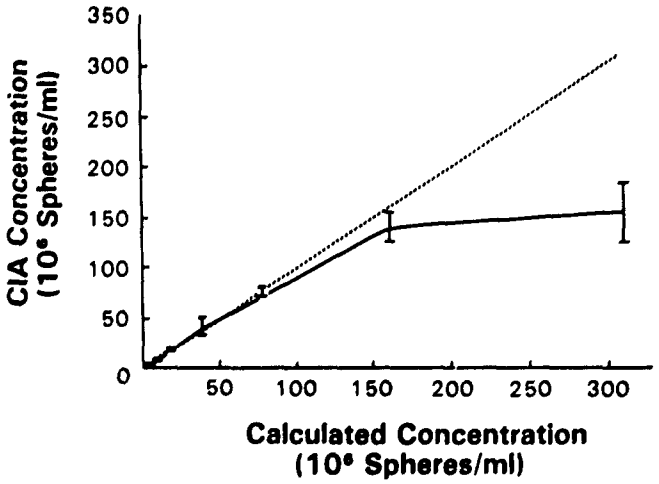

Fig. 8. A comparison of microsphere concentration determined by CIA (——) and theoretical calculation (. . .). A linear relationship ($r^2 = 0.98$) up to 1×10^8 microspheres/ml was found. CIA significantly underestimated the microsphere concentrations when the microsphere concentration exceeded this value. The reason CIA under-estimated the high microsphere concentrations is that there are many microspheres that overlay each other and are not counted. The display represents the mean ± standard error of the mean.

Another source of error is due to adjustment of the video camera. The SIT camera must be properly adjusted to prevent image bloom of microspheres because bloom increases the area of the microspheres. Image bloom could not be compensated when a higher than 50 watt mercury arc lamp was used.

A biological limitation of the analysis is that the carboxylated microspheres used have different surface properties from bacteria. It should be possible to modify this method and study receptor-mediated phagocytosis by CIA using antibody-coated fluorescent microspheres instead of uncoated microspheres. Bacterial hydrophilicity affects ingestion and/or digestion [29,30] and varying the surface hydrophilicity of the microspheres can be used to model resistance to phagocytosis. It also may be possible to use CIA to measure bacterial phagocytosis if fluorescent bacteria are used instead of microspheres. Because bacteria are significantly smaller than the microspheres used, high magnification would be required that would greatly reduce the rate of analysis. The asymmetric shape of the bacteria also would introduce errors in measure-

ments because the projected area is not the same for all orientations.

Although the CIA method excludes all microspheres not associated with a macrophage, the epi-fluorescence light microscope cannot determine whether microspheres are inside or attached to the outside of a macrophage. The number of microspheres on the outside of the cells was minimized by vigorously washing the cultures after phagocytosis, and inhibition of phagocytosis by cytochalasin B or low temperature (4°C) treatments showed that very few microspheres adhered to the cells (less than one microsphere per cell remained after washing). Nonetheless, occasionally an excessive number of microspheres remained after washing and these cultures were not analyzed. "Background" microspheres is defined as greater than 50 individual non-phagocytized microspheres per 1 mm^2, and/or microsphere aggregates having an area equal to or greater than a cell.

Confocal scanning fluorescence microscopy (CSFM), a new light microscope method, provides significantly higher resolution images of microspheres in phagocytes than conventional epi-fluorescence light microscopy due primarily to the exclusion of out-of-focus light, the major limitation in epi-illumination fluorescence microscopy [17]. Theoretically, using a CSFM as the imaging system for the CIA would provide more accurate phagocytosis data. CSFM can be used to optically section through individual macrophages to determine the three-dimensional distribution of microspheres within the cells. Knowing the three-dimensional distribution would eliminate the artifact caused by overlaying microspheres discussed above. In addition, combining CSFM with differential interference contrast (DIC) microscopy, microspheres attached to the outside of cells can be resolved from those inside the cells. CSFM would be particularly useful for phagocytosis analysis in cell aggregates. However, due to a significant decrease in the number of cells that could be analyzed because high magnification optical sections are needed, more time, storage space, and computer intensive processing would be required.

Since detailed quantitative morphological data of macrophages attached to substrate are needed to correlate function and structure, in the future, this method will be used with a confocal scanning fluorescence microscope. Nonetheless, CIA will be useful for screening the effects of chemicals on macrophage morphology, phagocytosis, and cell sub-classifications.

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