Evaluation of the Phagocytosis of Microspheres in V79 Cells by Flow Cytometry

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ABSTRACT. Phagocytosis of microspheres in V79 Chinese hamster lung cells was investigated by flow cytometry. Fluorescent microspheres (1.8 μm diameter) were used as the ingesta. Change in the number of V79 cells containing fluorescent microspheres was measured as an index of phagocytic activity. With time there was a sigmoidal increase in cells containing microspheres. The phagocytosis of microspheres is partially explained by two parameters introduced to describe the sigmoidal curves.

Phagocytosis has been studied by several methods: observation of cells by light and electron microscopy (7); measurement of the absorption and/or fluorescence of cells containing spectroscopically detectable material (6); measurement of the specific activity of radio isotopes incorporated into cells (1); and measurement of substances produced by enzymatic reactions triggered by phagocytosis (9). None of these methods, however, is suitable for the quantitative measurement of subpopulations of cells containing definite numbers of particles.

Flow cytometry has been used to observe the phagocytosis of fluorescent microspheres (11, 3, 4, 5). When cells containing such microspheres pass through a laser beam used as the excitation light, the fluorescence intensity of the individual cell corresponds to the number of microspheres it contains (11, 3). Using flow cytometry, we earlier reported on phagocytosis of fluorescent microspheres (1.8 μm diameter) in V79 Chinese hamster lung cells and showed a high correlation between our flow cytometric results and those of microscopic observations (3).

Now using flow cytometry we have investigated in greater detail the phagocytosis of microspheres in V79 cells. Changes in the subpopulations of cells containing microspheres were measured, the fraction of phagocytic cells being found to increase sigmoidly with time after microspheres additions to three cell populations of V79 cells; in balanced growth, arrested in the early S phase by hydroxyurea (HU), and in confluency.

MATERIALS AND METHODS

Cells. Chinese hamster lung cells (line V-79-B obtained from Dr. Yutaka Ishii, Osaka University) were maintained as growing monolayer cultures in Ham's F10 medium (Flow Laboratories) containing 10% fetal bovine serum (Flow Laboratories) in a humidified atmosphere of 10% CO₂ in air at 37°C. The cultures were routinely passaged at low density twice a week by splitting them at the ratio of about 1 : 20. The durations of the G₁, S and
G2 + M phases of these cells were 3.0, 4.0 and 1.5 h (2).

Cell Preparation. V79 cells in logarithmic growth were inoculated in Petri dishes (28 cm²; Nunc) at a concentration of 5 x 10⁴ cells/dish. These V79 cell samples were cultured for 12 h then washed once with HU medium containing 0.2 mM hydroxyurea (HU, Sigma). The cells were cultured again in HU medium for 6 h then released from it. When each cell sample was in the G₁ or G₂ + M phase, the medium was carefully replaced by HU medium. Fluorescent microspheres were added 5 h later, when the V79 cells in a dish were arrested in the early S phase, and the cell concentration was 2 x 10⁶ cells/dish.

Asynchronous V79 cells (2 x 10⁵ cells/dish) also were prepared by culturing them without HU treatment. Confluent V79 cells (about 5 x 10⁶ cells/dish) were prepared by inoculating Petri dishes with 1 x 10⁶ cells and culturing them for 24 h.

Treatment with fluorescent microspheres. Fluorescent microspheres (1.8 μm diameter, fluoresbrite, fluorescent monodisperse carboxylated microspheres; Polysciences Co.) previously treated with medium containing serum were added to each Petri dish containing V79 cells (2 x 10⁵ cells/dish). Experimental error because of microsphere sedimentation was reduced by shaking the dishes every hour. At various times, the cells were washed twice with PBS(–) (divalent, cation-free phosphate-buffered saline), treated with 0.3 ml trypsin-EDTA (0.17 % and 30 mM) for one minute at 37°C then suspended in 1 ml PBS(–). The fluorescence of the incorporated microspheres in the cells in suspension was measured immediately.

Fluorescence measurements. The fluorescence of individual V79 cells was measured with a Cytofluorograf 50H (Ortho Instruments) equipped with a 5W argon ion laser. The fluorescence (530 nm) and laser-scattering light (488 nm) from individual cells irradiated with focused laser light at 488 nm (250 mW laser power) were separated optically through an interference filter (530 nm peak and 20 nm band width) and a dichromatic mirror then detected with photomultiplier tubes. The integrated values of the signals from the photomultiplier tubes were adopted as the relative intensity of fluorescence from V79 cells instead of the peak height-signal intensities.

Measurements were repeated at least twice after rapid passage of 0.1 ml of each sample, and the results were recorded when there were no marked difference between data. Signals from cell doublets and free microspheres were eliminated electrically by the scatter window in the Cytofluorograf.

RESULTS

To examine changes in subpopulations of V79 cells phagocytizing fluorescent microspheres, we exposed three cell populations: one in balanced growth, one arrested in the S phase and one in confluence to fluorescent microspheres. The fluorescence of the microspheres present in the individual cells was measured by flow cytometry.

Representative example of data for relative fluorescence intensity (F₅₃₀) and laser-scattering light (S₄₈₈) from individual V79 cells are shown in Fig. 1. The upper panel gives the F₅₃₀-S₄₈₈ cytogram and the lower, the F₅₃₀ histogram obtained by selective counting within the window (indicated by the double arrow W in the cytogram). The numeral above each peak shows the number of microspheres in the cells that constitute that peak, as ascertained by sorting the peaks (3). The cell fraction for each peak was obtained by counting the cell number between the adjacent valleys in a histogram. The number of cells containing more than six microspheres were counted by integration within their region.

Changes in the proportions of cells containing microspheres are given in Fig. 2.
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Fig. 1. Representative data obtained from flow cytometry measurements of phagocytosis in V79 cells in balanced growth 6 h after a microsphere addition. Upper panel: the cytogram; the ordinate represents the relative intensity of scattered light ($S_{488}$) and the abscissa that of fluorescence ($F_{530}$). The origin is shown by “o”. In the cytogram, the two small populations close to the origin are free fluorescent microspheres; the subpopulations with large $S_{488}$ values are V79 cells. The lower panel represents the $F_{530}$ frequency distribution histogram obtained by selective counting of the cytogram region indicated by the double arrow, W. The numeral above each peak gives the number of microspheres in the cells that constitute that peak. The scale on the histogram abscissa shows the position of the valley between peaks.

Graphs A, B, C, and D in this figure show changes at the final microsphere concentrations of $1 \times 10^7$, $2 \times 10^7$, $4 \times 10^7$ and $8 \times 10^7$ microspheres/ml. The open circles represent measured values. Experimental errors for both the cell fraction and the time of measurement fall within the diameter of the open circles. Each open circle at a particular time represents the fraction of cells containing $p$ or more ($\geq p$) microspheres ($p=1, 2, 3, 4$ and $5$ in that order from the top). The cell fractions increase sigmoidly with time after the microsphere addition. Therefore, the fraction, $f_p(t)$, of
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Fig. 2. Changes in the fraction of V79 cells phagocytizing microspheres. V79 cells growing exponentially (about $2 \times 10^5$ cells/dish) were exposed to microspheres at a final concentration of $1 \times 10^7$, A; $2 \times 10^7$, B; $4 \times 10^7$, C; and $8 \times 10^7$ microspheres/ml, D. The open circles on the solid lines, $p$ ($p=1, 2, 3, 4$ and $5$) represent the fraction of cells containing $p$ or more microspheres. Each solid line was drawn by the least squares method using Eq. 1.

$$f_p(t) = \frac{(K_p t)^n_p}{1 + (K_p t)^n_p}$$ (1)

in which $K_p$ and $n_p$ are parameters.

Solid lines 1 to 5 drawn in Fig. 2 are the best fitting curves and were obtained by changing the parameters $K_p$ and $n_p$ in Eq. 1. Because these solid lines are within the margin of experimental error for each point, the increment in the fraction of phagocytic V79 cells clearly can be described by Eq. 1. Therefore, microsphere phagocytosis in V79 cells is characterized by the parameters $K$ and $n$.

Values of $K_p$ and $n_p$ for the solid lines in Fig. 2 are shown in Table 1. The values for $K_p$ increase with the increase in the microsphere concentration and decrease with the decrease in $P$. In contrast, $n_p$ increases with both the increases in microsphere concentration and $p$.

To remove any ambiguity in our characterization of phagocytosis by a cell cycle
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traverse of cells, we arrested V79 cells in the early S phase with HU. The change that occurred in the fraction of cell phagocytizing microspheres when a final concentration of $1 \times 10^7$ microspheres/ml was added to V79 cells arrested in the early S phase by HU is shown in Fig. 3. Change in this fraction also could be described by Eq. 1. Table 1 shows the $K_p$ and $n_p$ values, which are larger than their counterparts in A (same microsphere concentration).

<table>
<thead>
<tr>
<th>Cell population (microspheres/ml)</th>
<th>p=1</th>
<th>p=2</th>
<th>p=3</th>
<th>p=4</th>
<th>p=5</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ($1 \times 10^7$)</td>
<td>0.13</td>
<td>1.8</td>
<td>0.08</td>
<td>1.8</td>
<td>0.06</td>
</tr>
<tr>
<td>B ($2 \times 10^7$)</td>
<td>0.23</td>
<td>1.7</td>
<td>0.16</td>
<td>1.8</td>
<td>0.12</td>
</tr>
<tr>
<td>C ($4 \times 10^7$)</td>
<td>0.27</td>
<td>2.1</td>
<td>0.19</td>
<td>2.2</td>
<td>0.15</td>
</tr>
<tr>
<td>D ($8 \times 10^7$)</td>
<td>0.70</td>
<td>2.2</td>
<td>0.43</td>
<td>2.3</td>
<td>0.34</td>
</tr>
<tr>
<td>+HU ($1 \times 10^7$)</td>
<td>0.40</td>
<td>1.8</td>
<td>0.25</td>
<td>1.9</td>
<td>0.19</td>
</tr>
<tr>
<td>Confluent ($1 \times 10^7$)</td>
<td>0.79</td>
<td>2.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

A, B, C and D represent the cell populations A, B, C and D in Fig. 2, and +HU the cell population treated with HU (Fig. 3). Confluent refers to the cell population in confluency (Fig. 4).

Fig. 3. Changes in the fraction of V79 cells phagocytizing microspheres. V79 cells (about 2$\times$10$^4$ cells/dish) arrested in early S phase by HU treatment were exposed to microspheres at a final concentration of $1 \times 10^7$ microspheres/ml. The open circles on the solid lines, $p\ (p=1,\ 2,\ 3,\ 4$ and $5)$, represent the fraction of cells containing $p$ or more microspheres. Each solid line was drawn by the least squares method using Eq. 1.
A confluent arrangement of V79 cells should be physically restricted by cell-to-cell contact, which would limit the uptake of microspheres. Figure 4 shows changes in the fraction of confluent V79 cells phagocytizing microspheres. Each point given in the upper panel is the actual measured value. Although the fraction increases sigmoidly with time, none of these data points fit Eq. 1. In this upper panel, the fraction of cells that includes one or more microspheres reached a plateau at 0.9. This indicates that about 10% of the cells either were not in contact with the microspheres or could not phagocytize them. The lower panel in Fig. 4 shows the cell fraction corrected to account for this 10%. When $p=1$, each point is described by Eq. 1, but when $p$ has

![Graph showing changes in the fraction of V79 cells phagocytizing microspheres.](image)

**Fig. 4.** Changes in the fraction of V79 cells phagocytizing microspheres. Confluent layers of V79 cells (about $5 \times 10^6$ cells/dish) were exposed to microspheres at a final concentration of $1 \times 10^7$ microspheres/ml. The open circles at a particular time represent the fraction of cells containing $p$ ($p=1, 2, 3, 4$ and 5 from the top) or more microspheres. Broken lines were drawn to facilitate the reading. Each open circle in the upper panel shows the actual measured value, whereas those in the lower panel have been corrected for unexposed cells (10%). The solid line was drawn by the least squares method using Eq. 1.
a value other than 1, the points do not fit Eq. 1. The values for $K_1$ and $n_1$ when $p=1$ are larger than the values for $K_1$ and $n_1$ when $p$ has any other value (Table 1).

DISCUSSION

To determine the characteristics of the phagocytosis parameters $K_p$ and $n_p$, we drew simulation curves of Eq. 1, for which these parameters were varied (Fig. 5).

Fig. 5. Simulation curves for Eq. 1. The curves in the upper panel were drawn with $n$ kept constant ($n=2$) and $K$ varying from 0.1 to 1 at intervals of 0.1. The curves in the lower panel were drawn with $K$ kept constant ($K=0.2$) and $n$ varying from 1 to 3.
With a constant $n_p$ and an increasing value for $K_p$, $f_p(t)$ reaches half the maximum value in less time; i.e., altering the $K_p$ changes the time scale of phagocytosis. When $K_p$ is kept constant and $n_p$ increased, $f_p(t)$ reaches a plateau in less time. $K_p$ therefore appears to be related to the microsphere uptake rate and $n_p$ to the ease with which the next microsphere will be taken up.

The $K$ and $n$ values increased with the increase in the concentration of microspheres. This is an evidence that the phagocytosis of microspheres in V79 cells is a random process that permits cells to simultaneously take up more than one microspheres. The increments of $n_p$ with $p$ suggest that phagocytic activity of a V79 cell increases after the cell has taken up the first microsphere.

In V79 cells arrested in the early $S$ phase, the $K_p$ values are larger than those for cells growing exponentially at the same microsphere concentration. This is indicative of phagocytic activity in the early $S$ phase of V79 cells being higher than the average phagocytic activity throughout the cell cycle, or that HU increases phagocytic activity.

In a confluent layer of V79 cells, when $p=1$ $K_p$ was very large evidence of very high phagocytic activity, at least initially. By the fifth hour after the microsphere addition, however, microsphere phagocytosis had ceased; and by 10 h after the addition, the average microsphere number per cell was less than in cells arrested in the $S$ phase.

We conclude that in the phagocytosis of microspheres in V79 cells the fraction of phagocytic cells increases sigmoidly with time after the microsphere addition and that the change in the fraction can be described by Eq. 1, except when cell growth is confluent and phagocytosis is physically restricted. These results are valid for only this one type of V79 cells.

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