STRUCTURAL AND FUNCTIONAL STUDY OF ACIDIFICATION DURING PHAGOCYTOSIS

A. V. Khramtsov, V. V. Shcherbukhin, I. A. Morozov, A. V. Filatov, and V. M. Zemskov

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In the course of phagocytosis structural and functional changes are observed in the phagocytic cell. Structural changes in the phagocyte consist primarily of changes in its morphology: Initially a phagosome is formed; later, after its fusion with primary lysosomes, a phagolysosome [2]. Each of these stages is characterized by physicochemical action of the macrophage on the material to be phagocytosed. In particular, in the region of contact between plasma membrane and phagocytosed material protons are generated, leading to a considerable fall in pH [4].

The aim of this investigation was a quantitative study of acidification, using morphometric and cytofluorometric analysis of the kinetics of phagocytosis.

EXPERIMENTAL METHOD

Peritoneal macrophages activated by intraperitoneal injection of sodium caseinate into CBA mice 4 days before the experiment, were obtained by flushing out the peritoneal cavity with Hanks' solution containing 0.2% bovine serum albumin and 10 U/ml of heparin, and sedimented by centrifugation for 8 min at 400g, and the residue was resuspended in the same medium without heparin, and recentrifuged under the same conditions. Material for phagocytosis consisted of yeast cells (*Candida albicans*), killed by heat and opsoninized with rabbit antiserum. Phagocytosis was carried out at 37°C with constant shaking in medium containing 140 mM NaCl, 5 mM KCl, 20 mM HEPES (pH 7.2), and 5 mM glucose. The process of phagocytosis was stopped at different stages by addition of paraformaldehyde solution (final concentration 1.5%), and after sedimentation of the cells by centrifugation the material was subjected to standard treatment for electron microscopy [1]. The magnitude of the ultrastructural changes was estimated by means of stereologic transformations [5]. The material for phagocytosis in the cytofluorometric investigations was labeled with fluorescein isothiocyanate. Measurements were made on the 50-H cytofluorograph (Ortho Instruments). Fluorescence was excited at 488 nm by means of a 150-mW argon laser. The low-angle scatter of radiation of a helium–neon laser was recorded at the same time [3].

EXPERIMENTAL RESULTS

By transmission electron microscopy, direct analysis of images obtained by ultrathin sections is possible. During phagocytosis widely different complexes of phagocytic cells with adsorbed and ingested particles were formed. The thickness of the ultrathin sections was only 1/200 of the diameter of the phagocyte, and this explains the inadequacy of the distribution of the complexes observed in the electron microscope. Let the complex of a phagocyte with I ingested and J adsorbed particles be called an (I,J)-phagocyte, and let the section through the phagocyte containing S ingested and T adsorbed particles be called the (S,T) section. A mathematical method of creating the distribution of (I,J)-complexes on the basis of morphometric histograms of distribution of (S,T)-sections of these complexes was described in [5]. Table 1 gives the results of sterologic analysis of the kinetics of phagocytosis of *Candida albicans* cells by mouse macrophages. After 1 min 88% of cells adsorbed three particles on their surface, and by the 30th minute 75% of the phagocytes each contained five yeast cells. Incidentally, after 5 min more than 60% of macrophages had phagocytosed three or more particles. Most macrophages thus had ingested close to the maximal number of

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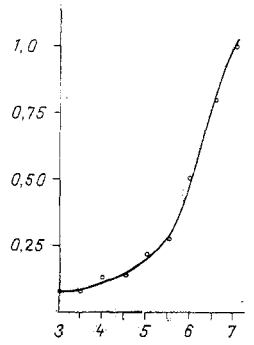

Fig. 1

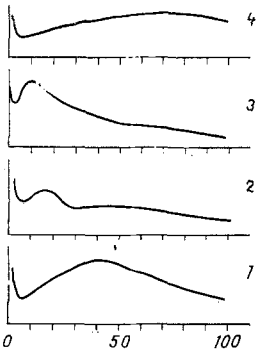

Fig. 2

Fig. 1. Effect of pH on intensity of fluorescence of yeast cells (*Candida albicans*), conjugated with fluorescein isothiocyanate. Abscissa, pH; ordinate, quenching of fluorescence, determined as ratio of intensity of fluorescence at the given pH to intensity of fluorescence at pH 7.0.

Fig. 2. Histogram of distribution of peritoneal exudate cells incubated with yeast cells (*Candida albicans*), conjugated with fluorescein isothiocyanate, by intensity of fluorescence, recorded after certain periods of incubation. Abscissa, intensity of fluorescence (in relative units); ordinate, number of cells (in relative units). Incubation for: 1) 1 min, 2) 10 min, 3) 30 min, 4) 30 min, followed by addition of 10 mm NH_4Cl.

particles after 5-10 min; the evolution of the distribution of the complexes agreed with the consecutive character of particle ingestion by the phagocyte.

The intensity of fluorescence of yeast cells conjugated with fluorescein isothiocyanate is largely dependent on pH [7]. The labeling procedure which we used yielded yeast cells with a high level of fluorescence. The shift of the maximum of the histogram of yeast cell distribution along the axis of intensity of fluorescence, depending on pH of the medium, is shown in Fig. 1. In the zone pH 3.0-8.0 the intensity of fluorescence changed approximately tenfold, and this is a sensitive indicator of the pH of the medium containing the yeast cells.

When acidification during phagocytosis was studied peritoneal exudate cells were incubated in the presence of a large (50-fold) excess of yeast cells. After certain time intervals samples were taken and analyzed on a continuous-flow cytofluorograph. For each histogram $20 \cdot 10^3$-$40 \cdot 10^3$ cells were counted. Yeast cells and peritoneal exudate cells differ considerably from one another in size, so that they could be differentiated by the degree of scattering of light, and fluorescence of the cells could be recorded separately. In this paper only the population of large peritoneal exudate cells is considered. The distribution of the cells by intensity of fluorescence at different moments of time is illustrated in Fig. 2. In the first sample, taken from the incubation medium 1 min after addition of the yeast cells, a monomodal distribution was observed, evidently due to phagocytes which adsorbed fluorescent particles on their surface. At the 10th minute of incubation a second peak appeared on the histogram in the region of a lower intensity of fluorescence, and probably due to a fall of pH in the newly formed phagosomes. In the next samples the intensity of the second peak rose progressively but the intensity of the first peak fell, and at the 30th minute of incubation a histogram with a single maximum was observed.

To analyze the cytofluorometric histograms (Fig. 2) by the aid of the data of stereologic analysis (Table 1), let us assume that quenching of fluorescence takes place after ingestion of particles with the formation of phagosomes (or phagolysosomes). In this case the intensity of F-fluorescence of the complex of a macrophage with I ingested and J adsorbed particles is determined by the equation:

$$F = Jqf + if,$$

where f is the intensity of fluorescence of a single yeast cell; q is the quenching factor of fluorescence of yeast cells in a state of phagocytosis.

TABLE 1. Kinetics of Phagocytosis of Yeast Cells by Activated Peritoneal Macrophages (X_{IJ})

Type of section (structure)		Time, min			
I	J	1	5	10	30
0	0	7	—	11	4
	1	4	13	—	—
	2	—	—	—	—
	3	88	—	—	—
1	0	—	—	—	1
	1	—	—	—	—
	2	—	26	—	—
	3	—	—	6	16
2	0	1	—	—	—
	1	—	—	—	—
3	0	—	—	0,5	—
	1	—	11	83	3
4	0	—	51	—	2
5	0	—	—	—	75

At the first minute 88% of macrophages adsorbed three particles, i.e., I = 0, J = 3 and the intensity of fluorescence was 40 relative units (Fig. 2). The equation obtained is thus 40 = 3f, from which the intensity of fluorescence of a single yeast cell can be calculated: f = 40/3 = 13.3. At the 30th minute most macrophages had ingested five particles, i.e., I = 5, J = 0, and the intensity of fluorescence was 10 relative units. These data give the second equation: 10 = 5qf, from which q = 0.15. Quenching of fluorescence, according to the calibration curve, thus corresponds to pH 4.5, in agreement with results obtained by other workers [6, 8], who used the ratio of intensities of fluorescence during excitation at two wavelengths as indicator of pH.

On addition of 10 mM NH_4Cl, which abolishes intracellular proton gradients [7], to the suspension of macrophages after incubation for 30 min with yeast cells, fluorescence of the macrophages rose sharply — from 10 to 75 relative units. This ratio of intensitites of fluorescence is in agreement with the quenching factor already calculated: q = 0.15. In fact, if the intensity of fluorescence of the (5,0)-complex without extinction was 75 relative units, in the presence of extinction the intensity would fall to 75•0.15 = 11 relative units, which was observed experimentally. Meanwhile, according to data in Table 1, at the 10th minute two peaks ought to be observed on the histogram of distribution of complexes by intensity of fluorescence: a large peak (83%), corresponding to (3.1)-phagocytes, in the position $F_1 = 3qf + 1f = 19$, and a small peak (6%), corresponding to (1,3)-complexes, with intensity of fluorescence $F_2 = qf + 3f = 42$. On the histogram (Fig. 2) peaks of corresponding intensity can be seen in positions $F_1 = 15$ and $F_2 = 45$. Complete mutual agreement is thus observed between data in Table 1 and the histograms.

In conclusion it may be pointed out that the combination of morphometry and cytofluorometry used in this investigation enables, on one hand, the acidification process to be recorded and, on the other hand, heterogeneity of the cell population by phagocytic activity to be evaluated quantitatively. The most complete and adequate idea of the structural–functional organization of the cell and its organelles can be obtained by a study of subcellular and molecular structures in the entire, intact cell, where all components are in natural interaction. The complex approach proposed in this paper considerably widens and deepens our opportunities in this respect.

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