IN VIVO FLUORESCENT PROBE STUDY OF ZYMOSAN-STIMULATED ALVEOLAR MACROPHAGES

L. N. Shishkina, N. G. Kolosova, and D. N. Mayanskii

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Stimulation of macrophages (M) is accompanied by structural changes in their membranes. Under the influence of active forms of oxygen and other biooxidants, accumulating in the phagosomes of cells during a "respiratory burst," intensive oxidation of arachidonic and other polyunsaturated fatty acids of membrane phospholipids takes place via the cyclo-oxygenase pathway, with the formation of prostaglandins and their derivatives, and via the lipo-oxygenase pathway with synthesis of leukotrienes and their derivatives [6, 8]. Meanwhile the topology and distribution of phospholipids in the membranes are drastically changed in activated M on account of acceleration of their resynthesis and breakdown [7]. Transformation of the phospholipid matrix of biomembranes is manifested primarily as a change in their viscosity. Accordingly M can be regarded as a convenient model with which to study correlation between function of the cell and the state of its biomembranes.

The aim of this investigation was to study viscosity and area of alveolar M (M_a) under normal conditions and after stimulation by zymosan granules (ZG) in vivo.

EXPERIMENTAL METHODS

Experiments were carried out on Wistar rats weighing 200-250 g. ZG were injected intravenously in a dose of 100 mg/kg, in a volume of 1 ml, 5 days before the investigation. Control animals were given an injection of 0.85% NaCl solution. The ingestive power of the mononuclear phagocyte system (MPS) was assessed as the rate of clearance of colloidal carbon (Gunter-Wagner, West Germany), injected intravenously in a dose of 20 mg/100 g body weight, from the blood. The number of M loaded with colloidal carbon was counted in lung sections in 10 fields of vision under a magnification of 1000 and in a total area of $14 \cdot 10^4$ μ^2. To obtain a suspension of M_a (bronchoalveolar lavage cells) the respiratory tract of animals of another group was compressed after decapitation, and rinsed out three times with medium 199 containing heparin [5]. After centrifugation for 15 min at 4°C and 2500 rpm the cells were resuspended in Hanks' medium without phenyl red, their number was counted, and they were used for determination of viscosity and area of the membranes [2, 3]. Pyrene and OSP-14 (pyridine derivative) fluorescent probes, spectral characteristics of which are given in [1], were used. They are localized mainly in the lipid phase of cell membranes. Parameters of fluorescence were recorded by means of an MPF-4 spectrofluorimeter (Hitachi, Japan) in a cuvette 0.5 cm in diameter at 20°C. The cell concentration was $2 \cdot 10^6$/ml. The viscosity of the lipid phase of the membranes was estimated from excimerization of pyrene [1]. The surface area of the membranes of M was measured, by recording the efficiency of migration of energy between pyrene, an energy donor, and OSP-14, an energy acceptor. On addition of OSP-14 to the M_a suspension, quenching of pyrene fluorescence takes place (Fig. 1). Having measured this efficiency, the total surface area of the cell membranes on one side of the bilayer can be calculated by the equation [3]:

$$\ln F_0/F \approx -\alpha \cdot C \cdot \frac{N_A}{S} \cdot B(x) \cdot \pi \cdot R_0^2,$$

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TABLE 1. Functional Characteristics of M_a under Normal Conditions and 5 Days after Stimulation by ZG in vivo (M ± m)

Experimental conditions	No. of M_a ($\times 10^6$) per gram of lung tissue	No. of M_a phagocytosing methacrylate granules, %	No. of M_a with diformazan deposits, %
Control (8)	3,44±0,46	12,7±0,59	10,2±1,5
ZG (8)	6,98±1,30**	28,5±1,18*	25,6±1,0*

Legend. *P < 0.01, **P < 0.05 compared with control. Number of animals given in parentheses.

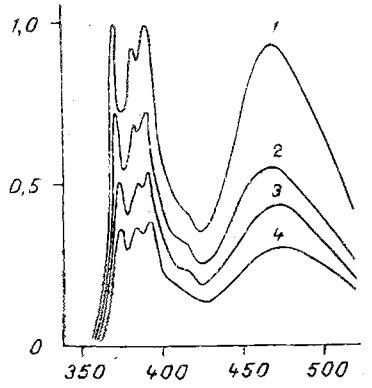

Fig. 1

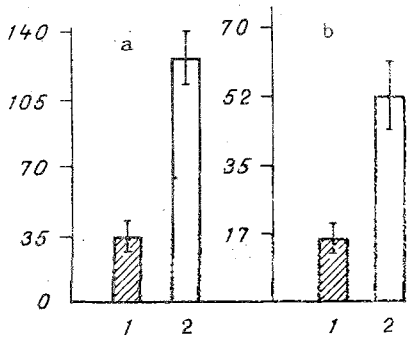

Fig. 2

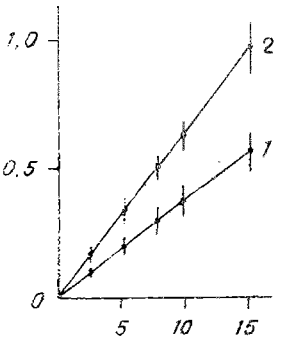

Fig. 3

Fig. 1. Quenching of pyrene fluorescence by probe OSP-14 in a suspension of M_a. Abscissa, wavelength (in nm); ordinate, intensity of fluorescence (in relative units). 1) Control; 2, 3, 4) OSP-14 in concentration of 6, 9, and 12 μm respectively. Number of cells $2 \cdot 10^6$ in 1 ml. Excitation at 334 nm, pyrene concentration 6 μm.

Fig. 2. Rate of clearance of colloidal carbon particles from blood (a) and number of M from interstitial tissue of lung, loaded with the same particles (b) in intact (1) and ZG-stimulated (2) rats. Ordinate: a) number of particles ingested per minute, b) number of M per $14 \cdot 10^4$ μ^2.

Fig. 3. Dependence of ratio F_e/F_m on pyrene concentration in M_a of control (1) and ZG-stimulated (2) rats. Abscissa, pyrene concentration (in μm). F_e and F_m) Intensity of fluorescence of excimers and monomers of pyrene respectively, measured at 470 and 272 nm. Cell concentration $2 \cdot 10^6$/ml, diameter of cuvette 0.5 cm.

where F_0 and F denote the intensity of fluorescence of pyrene before and after addition of OSP-14 respectively; α is a coefficient depending on the rotary movement of the probes, and has a value of 1.3 for the pyrene–OSP-14 pair; B(x) = 1.3 is a coefficient depending on the thickness of the membrane; R_0 is the Förster radius, namely 3.25 nm; C the concentration of OSP-14; S the surface area of the whole membrane on one side of the bilayer (in cm^2/liter); N_A is the Avogadro number.

Bronchoalveolar lavage cells were resuspended in 4 ml of medium 199 with 20% serum for nutrient media. The number of cells in 1 ml medium was $0.5 \cdot 10^6$. Coverslips with adherent cells were fixed with methyl alcohol and stained with azure-eosin; the number of M was counted and expressed in percentages. The cell suspension, in a volume of 0.5 ml, was introduced into polyethylene chambers mounted on slides, and allowed to stand for 1 h at 37°C.

Two identical monolayers were prepared from each animal: the nitro-BT test was carried out with one, and methacrylate granules (α = 0.9 μ, from the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague) were added to the other in the ratio of 100:1, the sample was incubated for 1 h and stained by Pappenheim's method, after which the number of phagocytic cells was counted in per cent.

EXPERIMENTAL RESULTS

Marked activation of MPS was observed 5 days after intravenous injection of ZG, as shown by several parameters. The velocity constant of clearance of colloidal particles from the blood and the number of interstitial M loaded with particles were both increased almost threefold (Fig. 2). The outflow of cells into the alveolar space was considerably increased. Their number reached $(11.8 \pm 1.7)\cdot 10^6$ per gram of lung tissue compared with $(4.3 \pm 0.6)\cdot 10^6$ per gram in the control. The main contribution to the increase in population was made by M_a (Table 1), including relatively small forms of M_a with a mean diameter of 11 μ. In their external appearance these cells resembled monocytes [5], but they possessed stronger adhesive properties. The number of M_a taking up methacrylate granules by phagocytosis was doubled, and the number reducing nitro-BT was increased by 2.5 times.

Investigation of the area of M_a of the control and ZG-stimulated rats revealed no difference: in the control it was $(25.5 \pm 0.9)\cdot 10^{-4}$ cm^2, in the experiment $(27.0 \pm 1.2)\cdot 10^{-4}$ cm^2 per cell. There was likewise no difference in the characteristic fluorescence of the membrane proteins. This suggested that the quantity of membranes in M_a was the same in the experimental and control animals. During investigation of viscosity of the lipid phase of membrane it was found that the efficiency of pyrene excimerization was significantly greater in M_a of the stimulated rats (Fig. 3). Since no differences were observed in the area of the membranes, the reason for this increase could only be the lower viscosity of the lipid phase of the membranes. The viscosity of the lipid phase of the membranes of M_a, calculated from the degree of quenching of fluorescence of pyrene monomers, was 1.1 $\pm$ 0.06 P in the control and 0.68 $\pm$ 0.03 P in the experiment (n = 25, $P < 0.001$).

Thus although the ZG-activated M_a have the same area of their membranes, they have a more "liquid" state of the lipids. Under these circumstances they possess higher clearing and bactericidal activity. On the whole, after stimulation by ZG, renewal of the M_a population is accelerated due to the inflow of monocyte-like cells, initially into the interstitial tissues, later from them into the lumen of the alveoli [4, 5]. Their high ingestive and bactericidal activity is combined with characteristic structural changes in the biomembranes, manifested ultimately as a reduction of microviscosity. it can be tentatively suggested that these changes in the physicochemical properties of M are an essential condition for the realization of its phlogogenic and bactericidal functions.

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