METHODS

Evaluation of Erythrocyte Shape and Status by Laser Interference Microscopy

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The area, thickness, and volume of erythrocytes of different types (discocytes, stomatocytes, and echinocytes) from normal subjects and coronary patients were studied by laser interference microscopy. Increase of pH value leading to the stomatocyte-discocyte-echinocyte transformations resulted in a slight decrease of cell volume. In coronary patients, erythrocyte had larger area and volume and exhibited increased aggregation capacity compared to erythrocytes from controls. The results recommend laser interference microscopy as an adequate method for erythrocyte evaluation in laboratory diagnostic measurements.

Key Words: *laser interference microscopy; coronary disease; erythrocytes*

Laser interference microscopy (LIM) is used for studies of reflecting, transparent, and semitransparent biological objects, for example, living cells and their organelles [1,4,7,9,13]. Alteration of erythrocyte shape and status is a diagnostic marker of various diseases [5,6]. Human erythrocytes are optically dense objects with homogenous contents, due to which their area, volume, and shape can be evaluated without staining.

Modern widely used automated hematological analyzers rapidly evaluate various characteristics of blood cells by their physical characteristics (*e.g.* volume or conduction), but this does not rule out the need in microscopic methods, because visual observation of the objects is often essential for accurate interpretation of the results. LIM allows visual observation of the cells and evaluation of their volume, which is an important advantage in comparison with traditional microscopy.

We studied morphological characteristics of various erythrocyte forms (discocytes, stomatocytes, echinocytes) and erythrocytes from coronary patients.

MATERIALS AND METHODS

Blood was collected from the ulnar vein into heparinized tubes, stored at 4°C, and used within several hours. Erythrocyte transition from normal discoid form into stomatocytes was induced by incubation in a buffer with pH 6.4, transition into echinocytes by incubation in a buffer with pH 8.4. Stomatocytes III and spherostomatocytes (the names of morphological forms correspond to the classification [5]) predominated at pH 5. Whole blood samples from 5 patients with different forms of coronary disease and from 1 donor were diluted (1:1) with plasma

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obtained by 10-min centrifugation of the blood. The sample was placed onto reflecting surface of a mirror slide and covered with a coverslip. The measurements were carried out at 18-22°C.

Erythrocyte shape, size, and volume were evaluated by LIM on a microprofilometer, designed at Institute of Optical and Physical Measurements (Moscow), on the basis of a MII-4 interferometer (LOMO). A 134×100.5 μ picture was registered. Phase image from the interference picture was reconstructed by the phase step method [12]. Laser (λ=650 nm, 5 mW power) served as the source of radiation, radiation power at the object was <2 mW. The solution and plasma refraction values, measured by IRF-454 BM refractometer at 18-22°C, were 1.335 and 1.340, respectively. Erythrocyte volume and shape were registered by LIM, the means were calculated from measurements of at least 70 cells per sample.

Cell height was calculated by the formula [7]. The coefficient of erythrocyte solution refraction was taken for 1.39 [2]. Horizontally lying erythrocyte conglomerations (more than 5 cells) were neglected in calculations.

RESULTS

Mature erythrocyte forms (discocytes, echinocytes, and stomatocytes) are uniform light-transmitting objects with known refraction coefficient, due to which their thickness and volume can be accurately evaluated by LIM [7]. Based on theoretical concept of cell thickness imaging by LIM [7], we hypothesized possible 3D phase images of erythrocytes of different morphological forms shown by this method. The thickness of a biconcave erythrocyte (discocyte) in the center is significantly lower in comparison with its edges in phase images (Fig. 1, *a*). Stomatocytes (cup-like cells) have a depression on only one side of the cell, while the other depression is leveled, but the erythrocyte is bended and looks like open mouth under light microscope [5]. Phase images of these cells are characterized by greater thickness and, as their structure suggests, lesser depression in comparison with discocytes (Fig. 1, *b*). Echinocytes are characterized by the presence of protrusions [5] locally increasing cell thickens (Fig. 1, *c*).

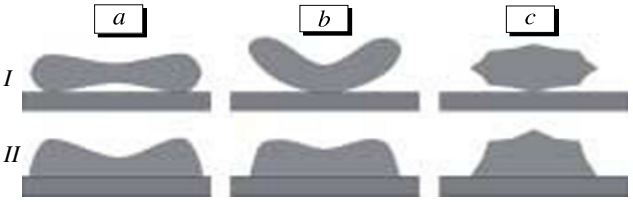

Fig. 1. Cross-sections (*I*) of a uniform discocyte (*a*), stomatocyte (*b*), echinocyte (*c*), and sections of their phase portraits (*II*).

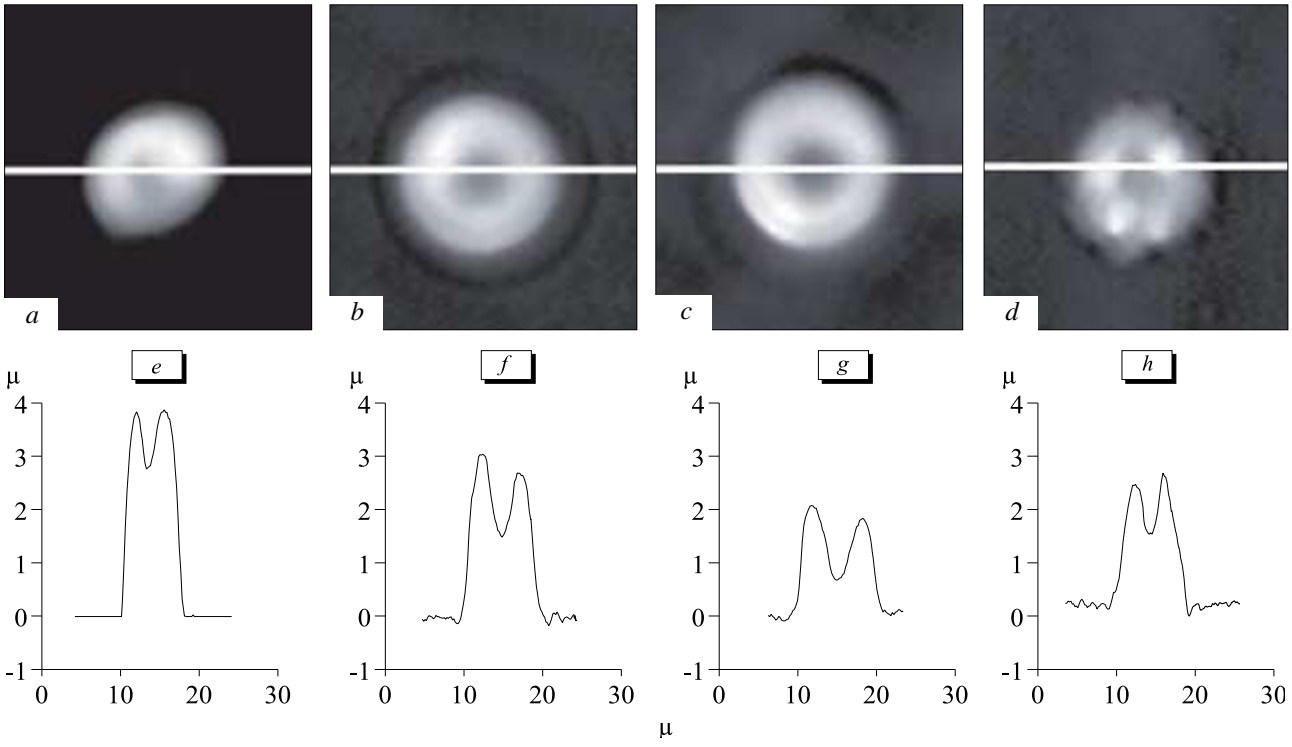

Fig. 2. LIM pictures (*a-d*) of various erythrocyte forms and changes in phase heights of phase profiles (*e-h*). *a*, *e*: type II stomatocyte; *b*, *f*: type I stomatocyte; *c*, *g*: discocyte; *d*, *h*: type I echinocyte. White line shows the direction of scanning during registration of the cell phase profile.

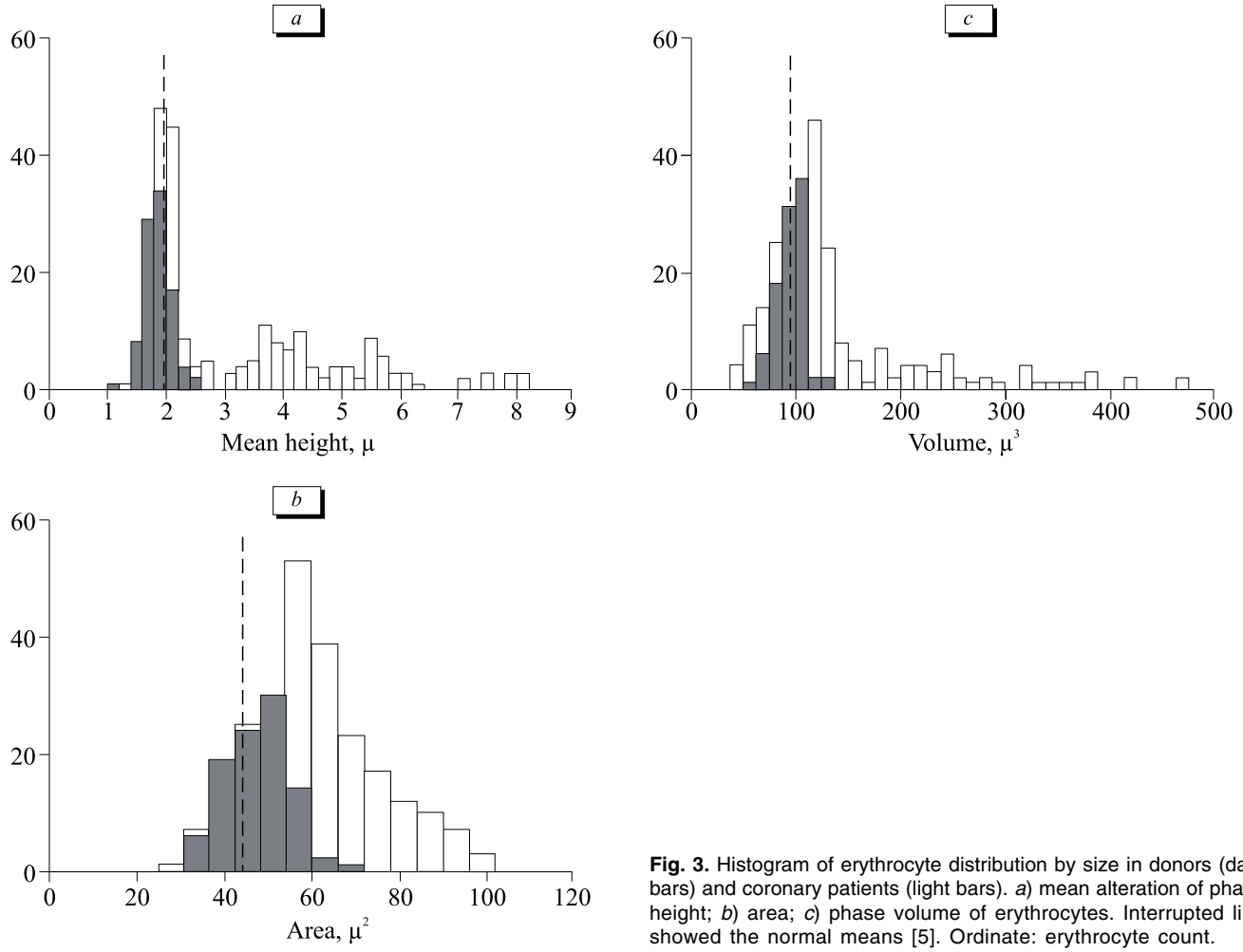

Fig. 3. Histogram of erythrocyte distribution by size in donors (dark bars) and coronary patients (light bars). *a*) mean alteration of phase height; *b*) area; *c*) phase volume of erythrocytes. Interrupted line showed the normal means [5]. Ordinate: erythrocyte count.

Changes in pH values can induce transition of discocytes (the most prevalent erythrocyte shape) into stomatocytes (acid pH) and echinocytes (alkaline pH) [5]. Comparison of phase images of erythrocytes obtained at different pH values (Fig. 2) with their presumable phase images (hypothesized from their structure) showed that experimental data corresponded to theoretically expected types of phase images.

Changes in optical density of erythrocyte cytoplasm were studied after cell incubation in media with different pH (Fig. 2). Transformation from type I-II stomatocyte into discocyte and then into type I-II echinocyte (corresponding to pH 6.4—7.4—8.4) is accompanied by a decrease in cell volume (135±10—103±2—100±1), which reflects the relationship between erythrocyte volume and shape [10] and confirms previous findings [12]. At pH 5.0, a decrease in the mean cell volume (96±3 μ^3) was observed during the formation of type III stomatocytes and spherostomatocytes.

We conclude from these results that LIM can be used for evaluation of various types of mature erythrocytes.

Comparison of erythrocyte sizes in donors and coronary patients showed that the mean volume, area, and height of donor erythrocytes corresponded to those described previously [6] (Fig. 3, *a*), while in coronary patients the means surpassed the normal values (Fig. 3). It is known that high aggregation capacity of erythrocytes in cardiovascular diseases can be caused by changed composition of the plasma and erythrocyte membrane [8]. The increase of erythrocyte area in coronary disease seems to be caused by high content of positively charged macromolecules in patient's plasma, which promotes cell adhesion to glass due to neutralization of negative charges on erythrocyte (glycocalix) and glass surfaces. However, erythrocyte membrane viscosity is also changed in coronary disease [3], and therefore we cannot rule our the effects of the membrane characteristics on erythrocyte adhesion to the sublayer and hence, on the erythrocyte area.

Erythrocyte distribution by height was polymodal in coronary disease, the mean values of the peak amplitudes being divisible by 2 μ (Fig. 3). Since erythrocyte height is about 2 μ, erythrocytes

aggregation and formation of rouleaux are possible, which corresponds to the data on high aggregation of erythrocytes in coronary and other cardiovascular diseases [8,14].

The mean volume of erythrocytes in coronary patients also surpassed the normal (about 125 μ). Erythrocyte rouleaux consisting of less than 5 cells "stood" vertically on the sublayer. It is easy to differentiate them from solitary cells by the LIM method (by the increase in the phase height) and thus obtain information about the formation of small erythrocyte aggregates, not discernible by common light microscopy.

The percentage of stomatocytes in coronary patients was 13% and of echinocytes 9%, which significantly surpassed the normal (3 and 6%, respectively).

Hence, increased number of erythrocyte rouleaux and intensive enlargement of erythrocyte area together with other symptoms can indicate a coronary disease.

These data persuasively indicate that LIM can be used for evaluating erythrocyte status, for example, for the diagnosis of coronary disease. The use of LIM provides information about linear size and volume of erythrocytes, their shape, and aggregation capacity. Moreover, the use of interference microscopes as a rule does not involve staining of the material, which appreciably facilitates manipulations with the preparations and accelerates the diagnosis. This recommends LIM for practical hematological laboratories.

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