Creation of New Physicochemical Methods for Blood Analysis on the Base of Fluorescent Probes

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This review deals with the development of new methods for studies of blood cells and plasma, based on the use of special dye molecules, the so-called fluorescent probes. These probes can also be used for clinical diagnosis. Probes and new methods on the basis of these probes were created for measurements of plasma and serum lipoproteins, serum albumin binding centers, blood leukocyte intracellular lipoproteins, allergens.

Key Words: *fluorescent probes; lipoproteins; albumin; leukocytes; allergens*

Physicochemical approaches to blood analysis

The organism is a system consisting of a great number of intermolecular interactions. Some of them eventuate in chemical changes in the molecules; these reactions are studied by biochemistry and organic biochemistry. However, the overwhelming majority of intermolecular interactions are caused by weaker, noncovalent forces and do not involve chemical restructuring. This fact does not at all diminish their role in the organism. It is sufficient to recollect such examples of "nonchemical" interaction as binding of two DNA chains, lipid-protein interaction with the formation of membranes, DNA-proteins interaction with the formation of chromosome, binding of antigens with antibodies, hormones and drugs with receptors, *etc.*

Physicochemical medicine (in its narrow meaning) studies disorders in these interactions underlying disease development and creates methods for their detection and correction. Study of these disorders leads to elucidation of molecular mechanisms of pathogenesis. Registration of these disorders will help to create new effective diagnostic methods. Correction of these disorders leads to creation of new effective therapeutic methods.

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Based on these common assumptions, new physicochemical approaches to blood analysis are developed at Institute of Physicochemical Medicine.

Fluorescent probes

Fluorescent probes (FP) are typical tools of physicochemical biology and medicine. These are molecules of special dyes binding noncovalently to cells, membranes, proteins, or other biological objects so that the fluorescence parameters of bound probe provide certain information on the structure and function of these objects. Two main characteristics of the probe are used: capacity to recognize certain sites in the protein, membrane, cell and capacity to fluoresce in a certain way depending on the physical properties of this protein, membrane, cells, *etc.*

A notable step in the development of FP method was the creation at the Second Moscow Medical Institute of the first uncharged FP, DMC and MBA [21] synthesized by S. V. Tsukerman (Kharkov State University) and by B. M. Krasovitskii and N. F. Levchenko (Institute of Monocrystals, Kharkov), respectively (Fig. 1). This was followed by creation of a group of new FP of different destination: DSM for studies of membrane potentials (synthesized by G. Ya. Dubur, Institute of Organic Synthesis, Riga), A6K and its derivatives (V. I. Shvets

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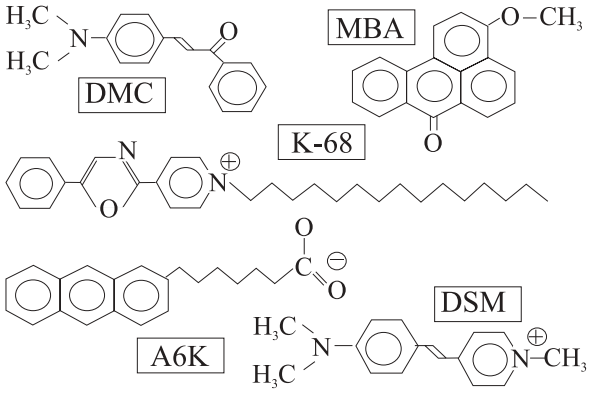

Fig. 1. FP for studies of cells, membranes, and LP, developed at Second Moscow Medical Institute and Institute of Physicochemical Medicine.

and A. S. Kaplun, Moscow Institute of Fine Chemical Technology), and K-68 (B. M. Krasovitskii *et al.*) for measurements of radiation-free energy transfer in membranes and lipoproteins (LP), K-37 for studies of LP, K-35 for studies of plasma albumin (B. M. Krasovitskii *et al.*), *etc.* At the beginning of 1980s Second Moscow Medical Institute and since 1983 Institute of Physicochemical Medicine became one of the leading centers of development of this effective physicochemical method for solution of problems of biomedical science [3,7]. At the end of 1970s the possibility of applying lipid and protein FP to clinical diagnosis was demonstrated at Central Research Laboratory of Second Moscow Medical Institute and then at Institute of Physicochemical Medicine.

Interactions of FP with blood plasma components

The new probes listed above were created for studies of blood cells and plasma components. The possibility to study physical characteristics of proteins and membranes in very small objects (for example, in a mitochondrion and, in principle, even in a protein molecule) and selective fluorescence of the probe only in certain biological structures is an important advantage of the FP method in comparison with numerous physical methods, involving an obligatory isolation of the studied protein from the plasma (or isolation of cells from the blood) and in great volumes.

Studies of the plasma provided the main results for LP and albumin.

Analysis of blood LP by FP. Cardiovascular diseases developing as a result of vascular atherosclerosis are the main cause of deaths in countries with well-developed economy. As is well known, the development of atherosclerosis is closely linked with cholesterol and triglyceride concentrations in the blood: their high concentrations are considered as serious risk factors for atherosclerosis development. Therefore screening of blood cholesterol and triglycerides and of LP carrying these lipids is obligatory and regular in countries with well-developed economy. Enzymatic methods in which serum samples are treated by several enzymes, are routinely used for this analysis.

Measures for prevention of atherosclerosis in the USSR in 1985-1991 necessitated the development of more simple, reliable, and at the same time cheap (in comparison with the enzymatic) screening methods for analysis. In order to solve this problem, a principally new method was designed at Institute of Physicochemical Medicine: addition of specially synthesized probe into the serum led to fluorescence of LP alone. The fluorescence intensity was proportional to serum cholesterol and triglyceride content. In 1989-1991 kits of reagents were manufactured and AKL-01 clinical fluorimeters were created, which were recommended by the Ministry of Health of the USSR for clinical use [13,14]. Unfortunately, the events that followed led to collapse of the program of blood lipid screening.

The prognosis of atherosclerosis development becomes more accurate if not lipids, but lipid-carrying LP are monitored. LDL deliver cholesterol to the cell and are assumed to be atherogenic, while HDL accept cholesterol from cells and are assumed to be antiatherogenic. VLDL are also atherogenic to a certain extent. Subsequent studies showed that the new FP fluoresced differently in different LP, because of different structure of their core. If fluorescence is stimulated very rapidly (within 2×10^{-10} sec) and its decay is recorded for 10^{-8} sec, analysis of the fluorescence decay curve shows individual LP concentrations [24,25]. It is particularly important for evaluation of the concentration of the most atherogenic LP (LDL). The data on LDL content obtained by the fluorescent method completely coincide with biochemical measurements (Fig. 2). The method is protected by International patents. At present the development of equipment for fluorescent express analysis of serum LP composition is at the stage of completion.

Hence, due to FP, alternative methods for the analysis of blood lipids and LP were created which have no analogs in the world.

Today many scientists are liable to think that the highest atherogenicity is associated not with total cholesterol fraction or LDL, but with the presence of small amounts of modified LDL (for exam-

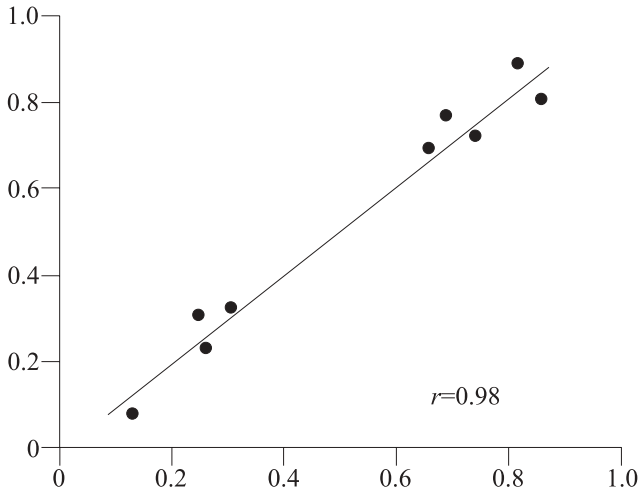

Fig. 2. Correspondence between biochemical (abscissa) and fluorescent (ordinate) data on the percentage of LDL in all serum LP. Fluorescent method is based on measurement of probe fluorescence decay in the serum.

ple, oxidized). The most pressing problem now is creation of simple methods for detecting modified LP. Presumably, FP will be useful for this analysis.

Serum albumin and its conformation in disease. Albumin amounting to 50% plasma protein has various functions. Albumin is the chief carrier of hydrophobic and amphiphilic low-molecular-weight substances: it transports fatty acids (the most important plastic and energetic substrate) to cells; delivers metabolites and xenobiotics (including drugs) to target organs and detoxification organs. Due to special binding centers for bivalent metals and highly reactive thiol in the albumin globule, albumin is the most important component of redox processes in the blood. Albumin is a source of bioactive oligopeptides, formed in degradation of this protein in tissues. The role of albumin as a specific inhibitor of endotheliocyte apoptosis was demonstrated.

Hence, albumin is an active component of numerous processes. However, presumably due to its importance, the concentration of albumin does not change in many diseases, remaining within the normal range. Because of this fact, physicians often neglect clinical biochemical characteristic "albumin concentration".

But if its concentration is so conservative, are the physicochemical properties of albumin molecule similarly conservative? The FP probe K-35 was designed to answer this question. Added into the serum, it binds virtually albumin alone, and the probe fluorescence increases by tens times. It is therefore possible to evaluate albumin directly in the plasma (serum) without its isolation.

In normal subjects K-35 fluorescence intensity was just proportional to its serum concentration (Fig. 3). In disease the fluorescence is significantly lower (sometimes several times lower) than with albumin molecule in its "native" state.

Study of the causes of this drop in fluorescence level in disease showed that the conformations of albumin molecule vary even when the total concentration of albumin is normal. These conformation changes are detected by K-35 [6,15]. The probe is located at the site of albumin molecule where albumin binds (noncovalently) fatty acids and drugs. The conformation of protein molecule is modified during binding of fatty acids or other ligands [1]. For example, this is the case in mental diseases, when the majority of biochemical values of the blood (including albumin concentration) are as a rule within the normal range.

Kits of reagents were created in 1994 for wide clinical studies of this reaction to a pathological process. The fluorescent test is rapid, requires only 20 μl blood, and the analysis takes 1 min. Due to this the studies were carried out in more than 60 clinics of the country on more than 20,000 blood samples [1].

It was found, among other things, that albumin fluorescent test was prognostically significant in surgical suppuration. A multicenter study of the significance of albumin test in diffuse peritonitis was therefore carried out. Combination of two parameters (effective concentration of albumin, ECA and urea concentration in the blood) during the first 24 h postoperation predicted the development of peritonitis with high probability (Fig. 4); no other objective parameters or clinical scores could predict it with so high probability [4,5,18]. In acute pancreatitis albumin test indicated the disease form,

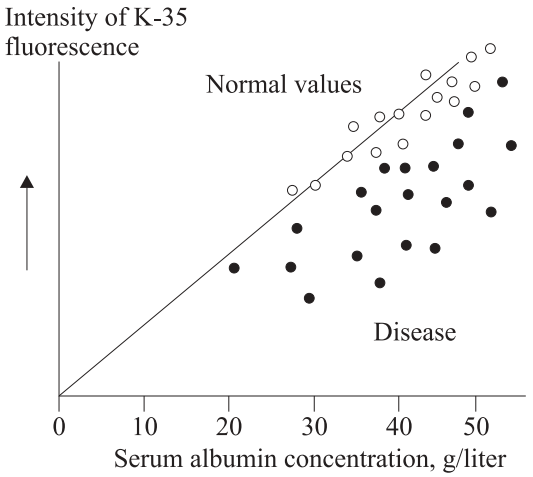

Fig. 3. Intensity of K-35 fluorescence in normal donor sera (light circles) and sera of patients with various diseases (dark circles).

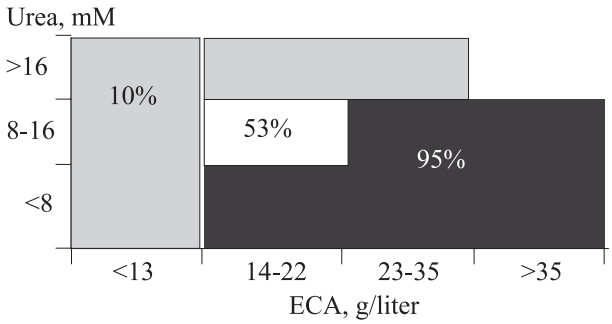

Fig. 4. Prediction of survival (%) in diffuse peritonitis determined 24 h postoperation by a combination of two parameters: albumin (ECA) and urea concentration in the blood.

severity, and prognosis [11,20]. The test is useful in critical states [5]. Interesting data on reparative processes after myocardial infarction were obtained [2,22]. Stress [19] and its aftereffects [26], as well as mental disorders [16,18] are detected by this method. Study of albumin conformation in tears and aqueous humor of the eye indicate the diagnostic and prognostic potentialities of this test in ocular diseases [12].

Hence, changes in the conformation of albumin binding centers can be an important factor in the pathogenesis of many diseases [6]. There are good grounds to assert that modification of albumin conformation is a new type of reaction to a pathological state, highly prevalent in many diseases.

Interactions of FP with blood cells

The cells have been studied with the use of stains for more than 200 years. The results of cell studies are becoming better clear during recent 30 years, due to development of variants of the FP method. Two new fluorescent methods for life-time studies of white blood cells were developed at Institute of Physicochemical Medicine.

Intracellular LP. The most obvious manifestation of atherosclerosis is appearance of cholesterol plaques on the vascular walls. A great number of lipids in the form of lipid droplets inside the plaque cell looks like a foam ("foamy" cells). Lipids get into the cell from the plasma mainly in the form of LDL. Therefore blood lipid level correlates to a certain measure with the appearance of cholesterol plaques. Unfortunately, this correlation is not sufficiently strong, and the accuracy of prediction of atherosclerosis course by plasma lipids is low.

It is important to measure the level of intracellular LP, but only methods for measuring extracellular (plasma) LP are available for clinical studies. No convenient object, which could indicate the level of intracellular LP, was found. Blood cells are a convenient object, but erythrocytes contain no intracellular LP, while in leukocytes no LP could be reliably detected up to recent time.

In order to clear out whether LP are present in leukocytes, two forms of lipid packing had to be distinguished: it is obvious that the greater part of lipids forms membranes, but some part of them is presumably packed in LP. Special technology for measuring radiation-free energy transfer [7] between DMC and K-68 was used for this purpose (Fig. 1). It was found that the leukocyte granulocytic fraction contained just about 10% LP of the total cellular lipid content, and hence, this very low content could not be detected before; lymphocytes contained no LP [27,28].

At present the location of LP in the granulocyte and relationship between the content of these intra-

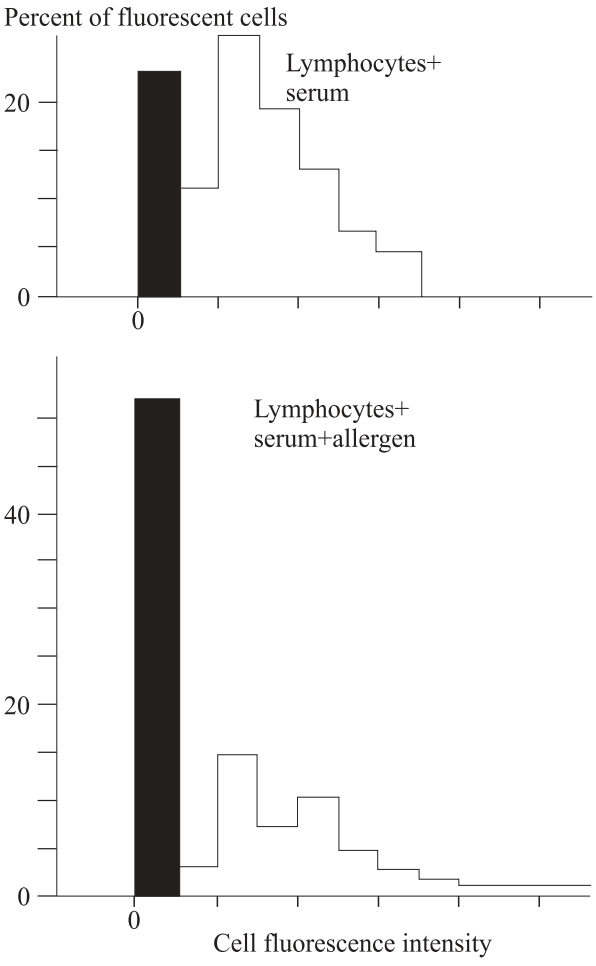

Fig. 5. Decrease of lymphocyte fluorescence intensity in the presence of serum from a pollenosis patient after addition of the allergen to which the patient is hypersensitive. Histogram of cell distribution by intensity of fluorescence of the DSM potential-sensitive probe. Dark bar: percentage of virtually nonfluorescent cells.

cellular LP and degree of atherosclerosis development are investigated.

Transmembrane potentials of cells and their changes in disease. A fluorescent method was developed at Institute of Physicochemical Medicine, due to which the electric field potentials on two lymphocyte membranes (plasma and mitochondrial) were for the first time measured in living cells [8-10]. A charged FP DSM (Fig. 1) was created for this purpose [29].

This method was used for the development of an original method for the diagnosis of immediate type hypersensitivity to pollen allergens. It was found that addition of serum from an allergic patient to test lymphocytes did not lead to changes in the electric field potentials, while if a patient was sensitive to some allergen, addition of this allergen to patient's serum sharply reduced transmembrane potentials [30,31]. This manifested in a drop of fluorescence intensity of DSM accumulated inside the cells due to electrical fields on the membranes (Fig. 5). The advantage of the method is the possibility of *in vitro* testing, in contrast to provocation tests in the patients.

Tumor-associated antigens also modify electric fields of patient leukocytes, which is a potential approach to the search for new methods for early diagnosis of cancer [23].

Hence, FP are effective tools for studies of physical characteristics of plasma cells and components, in contrast to the majority of biochemical (or hematological) methods, oriented to measurements of substances or cell counts and do not characterize the physical status of molecules or cells. Information about physical status of blood components (electrical fields of cells, protein conformation, structural organization of LP, *etc.*) is closely connected with the development of a pathological process. Further development of physical methods for blood analysis, including the FP method, will provide additional information about the patient, which is sometimes more useful than the information obtained by the traditional clinical tests.

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