A NEW TECHNIQUE FOR STUDYING PLATELET AGGREGATION IN VITRO

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Turbidometry, suggested in 1962, is the method most widely used to study platelet aggregation in vitro [2]. This method continuously records the transmission of light by a mixed sample of platelet suspension. Born's method [2] is frequently used as the investigative technique, but the transmission of light by a platelet suspension depends on a considerable number of parameters to which sufficient consideration may not be paid: the shape of the platelets, absorption of light by the blood plasma, distribution of the aggregates by size, and so on [3, 5, 8]. There may be cases when two specimens differ in the number of platelets forming aggregates and in the size of the aggregates formed, but at the same time they have equal optical density, i.e., transmission of light does not unequivocally reflect the size of the aggregates [7].

In the present investigation a new method was used to study platelet aggregation in vitro, by which the process of platelet aggregation can be characterized more precisely and completely.

EXPERIMENTAL METHOD

To investigate platelet aggregation we have developed a method involving the creation of a flow of cells through an optical channel and analysis of fluctuations in the intensity of light transmitted through the suspension. The optical channel of a standard Born aggregometer usually accommodates nearly the whole volume of the suspension and the conditions do not permit generation of a flow of particles through the optical channel. The main cause of appearance of fluctuations in the intensity of transmitted light under these circumstances is rotation of nonspherical particles in the flow. If a thin collimated pencil of light is used and the specimen is mixed, different numbers of platelets will enter the optical channel at each moment of time. This type of particle flow obeys the Poisson distribution [1]. For such a distribution, dispersion of the deviation of the number of particles from the mean will be equal in value to the mean. Changes in the number of particles in the channel will cause fluctuations in the intensity of light passing through the specimen. In the real instrument superposition of the above causes of the appearance of fluctuations of light passing through the cell suspension will be observed. However, the frequency spectrum of signal fluctuations caused by a change in the number of particles in the optical channel occupies a much greater band than the spectrum of oscillations connected with rotation of platelets in the flow. Thus these signals can be effectively separated from one another by means of frequency filters (Fig. 1). The intensity of light transmitted through a suspension of platelets and their aggregates depends exponentially on the extinction of the specimen [6]:

$$I = I_0 \cdot \exp\left[-l \cdot \sum_{n=1}^{\infty} S_n \cdot K_n \cdot N_n - E_0\right],$$

where I_0 is the intensity of incident light, S_n the area of cross section of aggregates of n platelets, K_n the effectiveness of scattering factor, N_n the number of aggregates of n platelets, l the length of the optical path, and E_0 the extinction of the medium.

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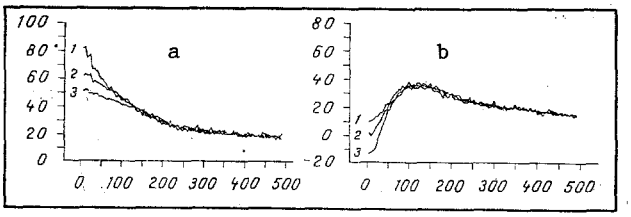

Fig. 1. Power spectra of fluctuations of light transmission by a mixed suspension of particles. a: Erythrocytes (1), platelets (2), and spherical latex particles 5.33 μ in diameter (3); b: the same spectra obtained by the use of a second order high-frequency filter with cutoff frequency of 100 Hz. Spectra obtained by fast Fourier transform program on a Labtam-3015 computer. Abscissa, frequency (in Hz); ordinate, spectral density (in dB).

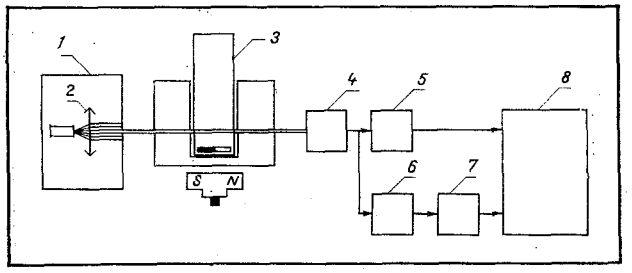

Fig. 2. Block diagram of experimental system (explanation in text).

This relationship is satisfied if the light is scattered once. In a platelet suspension, for visible and infrared light and with an optical path length of 5 mm, the condition of single scattering of light is satisfied at concentrations of up to 500-600 thousand platelets/μl. Platelet-enriched plasma, obtained by standard methods, certainly satisfies this condition. When a flow of particles is created their number in the optical path is described, as was stated above, by a Poisson distribution. The relative dispersion of fluctuations of intensity of light transmitted by the suspension will be described by the equation:

$$D = \frac{3}{4} \cdot \pi \cdot l^2 \cdot V_0 \cdot \varphi \cdot \frac{\sum_{n=1}^{\infty} \frac{K_n^2}{F_n} \cdot R_n \cdot n \cdot N_n}{\sum_{n=1}^{\infty} n \cdot N_n},$$

where R_n is the radius of an aggregate of n platelets; F_n the fraction of the volume of an aggregate of n platelets actually occupied by platelets; V_0 the volume of the optical channel; φ the volume concentration of platelets in the suspension. The equation thus obtained shows that the relative dispersion of fluctuations is independent of the intensity of incident light and of the light-absorbing activity of the medium. Moreover, this parameter is proportional to the weighted mean radius of the aggregates, where the weighting factor has the value K_n^2/F_n and the averaging is done with respect to volume distribution of the platelets and their aggregates according to size. If the volume concentration of the platelets in suspension is constant, a change in the relative dispersion of fluctuations will be due to an increase or decrease in the mean optical radius of the aggregates. Taking account of the propositions mentioned above, we have designed and built the system for which a block diagram is given in Fig. 2. A semiconductor emitter 1 with collimating optical system 2, used as the source of light, creates a narrow beam of light, so that the optical channel accommodates only a very small part of the volume of the sample 3. The intensity of light passing through the specimen is converted by the light receiver 4 into an electric signal, which is led to the input of the processing block. There it passes through a low-frequency filter 5, isolating its constant or, more exactly, its slowly changing component I. The same signal is also led to a high-frequency filter 6,

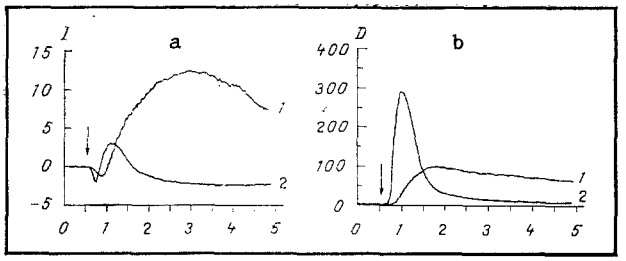

Fig. 3. Aggregation of human platelets induced by ADP and PAF. Abscissa, time (in min); ordinate: a) transmission of light I (in %); b) relative dispersion of fluctuations of transmission D (in relative units). 1) ADP (0.5 μM), 2) PAF (5 nM). Arrow indicates time of addition.

and from it to a device measuring the true mean-square value of the signal 7. The high-frequency filter isolates fluctuations of light transmission caused by a chaotic change in the number of particles in the optical channel and suppresses fluctuations caused by rotation of nonspherical platelets in the flow. After preliminary processing the signals are led through an ADC into the computer 8, which performs the subsequent calculations. The mean square value of the fluctuations of light transmission is divided by I, and the value thus obtained is squared. The relative dispersion of fluctuations of light transmission D, thus calculated, was used as the parameter for estimating the degree of platelet aggregation. The intensity of light transmitted by platelet-deprived plasma (PDP) was taken as 100%, and through platelet-enriched plasma (PEP) as 0%. The relative dispersion of fluctuations of light transmittance D recorded before addition of the inducer was taken as I. Taking the volume concentration of platelets in PEP as 0.25%, this value corresponded to about $(2\text{-}3) \times 10^{-6}$. Blood was obtained from donors by puncture of the cubital vein and stabilized with a 0.13 M (pH 7.4) solution of sodium citrate in the ratio of 9:1. The PEP was prepared by centrifuging blood at 200g for 9 min. PDP was obtained by centrifuging PEP at 2000g for 15 min. The volume concentration of platelets in PEP was determined by centrifugation, as described previously [4], and adjusted to 0.25% by mixing the appropriate volumes of PEP and PDP. The following preparations were used: ADP and platelet-activating factor (PAF) from "Serva" (West Germany); latex particles from "Polysciences Inc." (USA); other reagents were of the chemically pure grade.

EXPERIMENTAL RESULTS

The suggested method was used to investigate the kinetics of ADP- and PAF-induced aggregation of human platelets. So that results obtained with the use of different specimens of cell suspensions could be compared, all curves were drawn for volume concentrations of platelets in PEP of 0.25%. Simultaneous recording of light transmission of the specimen and the relative dispersion of fluctuations of transmission showed that a change in the transmission of light did not correlate with the change in the mean optical radius of the aggregates, whatever the concentration of the inducer. The maximal value of the radius of the aggregates was reached considerably earlier (from 10-20 sec with low exposure doses to several minutes for high doses) than the maximum of transmission. This result is in good agreement with data obtained by other workers using the "Coulter Counter" method [7].

Typical curves obtained after addition of 0.5 μM ADP are illustrated in Fig. 3. Addition of the inducer to the suspension caused a change of shape and aggregation of the platelets. This was accompanied by a decrease, and than an increase in light transmission (reversible, see Fig. 3a, curve 1). As regards the mean radius of the aggregates, it reached its maximal value, recorded as D (see Fig. 3b, curve 1), almost 1 min earlier than light transmission reached its maximum.

The change in light transmission during platelet aggregation did not correlate with D, not only in the time of reaching its maximum, but also in the amplitude of the effect. By way of demonstration, Fig. 3 shows the results of an investigation of PAF-induced platelet aggregation. Addition of PAF in a final concentration of 5 nM is indicated by an arrow. The greatest value of the mean radius of the aggregates (Fig. 3b, curve 2) after treatment with PAF was three times higher than after addition of ADP. Meanwhile the degree of ADP-induced platelet aggregation, determined by Born's method, was almost three times less than after addition of PAF.

The suggested method thus provides information about the process of platelet aggregation which differs radically from that obtained by the standard turbidimetric method. This information will be very useful both for scientific research and for the diagnosis of changes in the aggregating activity of platelets during the development of various diseases.

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PHOTOGENERATION OF SINGLET MOLECULAR OXYGEN BY COMPONENTS OF HEMATOPORPHYRIN IX DERIVATIVE

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The method of photodynamic therapy (PDT) is based on photodestruction of tumors, the process being sensitized by molecules of porphyrins selectively accumulated in cancer cells [5]. The active fraction of "hematoporphyrin IX derivative" (HPD), known commercially as "Photofrin II," is the agent most widely used in clinical trials at the present time [15]. HPD is a complex mixture of variable composition of products of alkaline treatment of acetylated hematoporphyrin IX, and includes porphyrin monomers: hematoporphyrin IX (HP), hydroxyethylvinyldeuteroporphyrin IX, protoporphyrin IX, and their dimeric and oligomeric derivatives; the phototherapeutic effect, moreover, is determined by these di- and oligomeric fractions. Photofrin is relatively richer in polymeric fractions than HDP but also contains a mixture of the above compounds [5, 15]. Previous investigations [2, 3, 6, 8, 10, 11, 13] have shown that if solutions and cells stained with HP, HDP, and Photofrin are illuminated, excited oxygen molecules in the $^1\Delta_g$-state ($^1O_2$) which appear when energy is transferred to O_2 from triplet porphyrin molecules, are formed. This led to the idea that it is $^1O_2$ which is the principal cytotoxic factor responsible for the action of these preparations [5]. However, it must be noted that $^1O_2$ generation has been investigated only in specimens containing HP or the total of all components of HDP, whereas activity of the polymeric components, responsible for the phototherapeutic effect, has not been successfully investigated. The technique of separation and purification of monomeric, dimeric, and oligomeric components of HDP which have been developed [12] has made the experimental study of this problem possible.

In the investigation described below a direct luminescent method of recording singlet oxygen [9] was used to determine the efficiency of $^1O_2$ generation by dimeric (DF) and oligomeric (OF) fractions of HDP, and also by purified HP, under conditions simulating their state in cellular solutions and cell membranes.

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