25-30 min, i.e., the saving of time amounts to 10-15 times. The method also is much less laborious, but at the same time it increases the accuracy and objectivity of the investigations.

Thus although the manual method of analysis of microfilms can yield several important characteristics of blood flow structure under various physiological and pathological conditions, it has important disadvantages, namely the great laboriousness of analysis (interpretation) of the frames and the possibility of subjective assessments of the results of the measurements. Moreover, with manual analysis the available information on flow structure is not fully utilized. The program of automatic frame-by-frame interpretation of microfilms which we have developed not only makes it possible to analyze them quickly and qualitatively, but also yields a greater number of parameters reflecting the behavior and interaction of red blood cells moving in microvessels.

LITERATURE CITED

1. V. A. Mamisashvili, B. S. Solov'ev, and Yu. I. Levkovich, Fiziol. Zh. SSSR, **68**, No. 6, 832 (1982).
2. V. A. Mamisashvili, I. K. Baratashvili, and D. G. Lominadze, Izv. Akad. Nauk Gruz. SSR, Ser. Biol., No. 3, 197 (1984).
3. G. I. Mchedlishvili, V. A. Mamisashvili, and M. N. Varazashvili, Patol. Fiziol., No. 6, 75 (1985).
4. V. S. Shinkarenko, Patol. Fiziol., No. 1, 64 (1983).
5. G. Bugliarello and T. W. Hayden, Trans. Soc. Rheol., No. 1, 64 (1983).
6. P. Gaehtgens, H. T. Meiselman, and H. Wayland, 25th Eur. Conf. Microcirc., H. Harders (ed.), Basel (1969), pp. 571-578.

MICROSPECTROFLUOROMETRIC ANALYSIS OF THE ACTION OF MECLOFENOXATE ON LIPOFUSCIN GRANULES OF HYBRIDOMA (RETROVIRUS-TRANSFORMED) CELLS

A. B. Tatariunas

UDC 578.242.4:578.1:577,115].04:[615.214.32:577.175.12

Key words: lipofuscin; meclofenoxate (centrophenoxine, acephen); hybridoma culture.

It is now almost 30 years since attention was first drawn to the stimulating action of a newly synthesized compound ANP-235, a derivative of the natural plant growth factors (auxins) on the vertebrate CNS [11]. This substance, an ester of dimethylaminoethyl-*p*-chlorophenoxyacetic acid, was later called meclofenoxate (MF), with synonyms centrophenoxine, acephen, lucidril, cerutil, etc.

MF lowers the intracellular potassium concentration [14], increases the rate of synthesis of total and mRNA [15], increases the density of adrenergic receptors [12], influences the adenylate cyclase system [9], acts on phospholipid metabolism [6, 8], and modifies the lipid composition of the cell membrane so that it becomes more fluid [13].

This perturbation in the lipid composition of the cell membrane is naturally reflected also in the state of intracellular structures. There is evidence that MF reduces the number of lipofuscin granules (aging pigment) in cells both during natural

Central Research Laboratory, Kaunas Medical Institute.

(Presented by Academician of the Academy of Medical Sciences of the USSR J. J. Bredikis.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 109, No. 4, pp. 345-348, April, 1990. Original article submitted March 5, 1989.

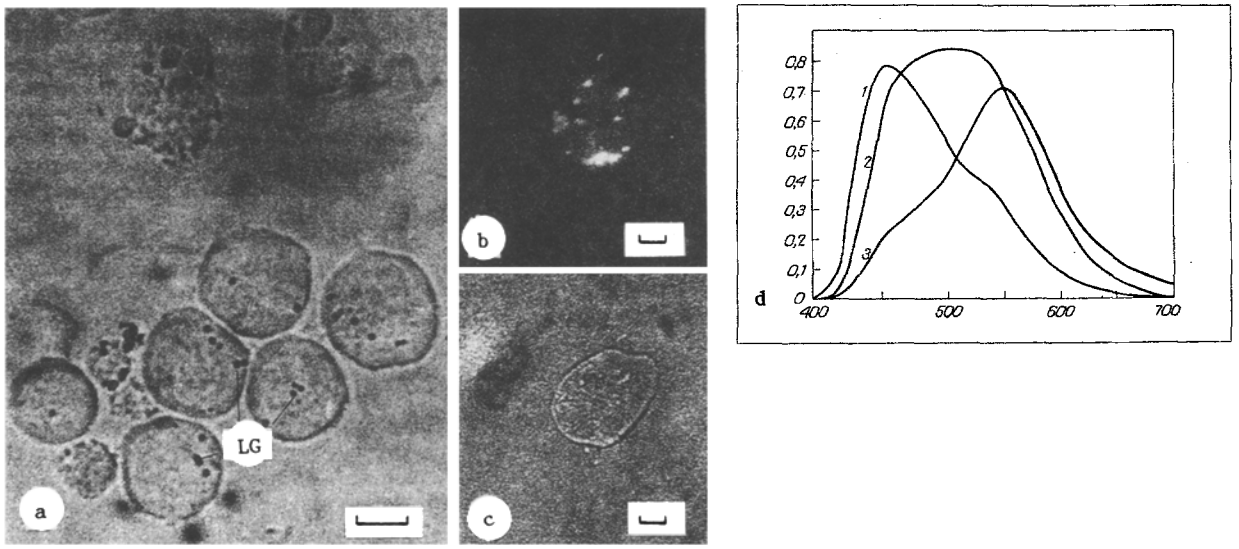


Fig. 1. LG in hybridoma cells during special microscopic examination in transmitted light (a) and in light of natural luminescence (b, c — outline of this cell), and spectra of natural luminescence of different clusters of LG (d). 1) Spectrum with distinct maximum in 440-460 nm region, 3) the same in 540-560 nm region, 2) luminescence spectrum when maxima in these regions were about equal in intensity. Abscissa, wavelength (in nm); ordinate, intensity of luminescence (in relative units). Scale 10 μm.

aging and during simulation of the aging process in culture [5, 7]. As a rule the physiological effect of MF is assessed by the number of lipofuscin granules (LG) in cells. However, the existence of natural luminescence of LG and the relative simplicity of their identification through this parameter [3, 4, 10] mean that it can be used to analyze the action of MF on living cells by a microspectrofluorometric method. Further scope for this kind of analysis may result from the effect of an increase in the intensity of the natural luminescence of LG in response to ultraviolet (UV) irradiation, which the writer discovered in cells [3, 10] and later confirmed on isolated LG [4].

It was accordingly decided to study the kinetics of the response of LG to the action of exciting UV on native retrovirus-transformed cells cultured in the presence and in the absence of MF.

EXPERIMENTAL METHOD

Hybridomas were obtained by fusion of Sp2/o-Ag14 mouse myeloma cells with spleen cells of Balb/c mice immunized with bacteriophage λ. They were cultured by the method described previously [2]. Subculture was carried out after 3-4 days. The MF was dissolved in culture medium and a fresh portion was added daily in a concentration of $5 \cdot 10^{-4}$ M.

ML-3 and LYUMAM-IZ luminescence microscopes (Leningrad Optico-Mechanical Combine) were used for microscopic investigations of the hybridoma cells. For luminescence analysis of LG a microspectrofluorometer with variable wavelength interference filter [1] was used. Luminescence of LG was excited by radiation λ = 365 nm from a DRSh-250-2 mercury arc lamp, separated by UFS-6 filter. The diameter of the microspectrofluorometric probe was 7 μm. The hybridoma cells were studied with the aid of a water-immersion luminescence 85 objective in a drop of culture medium or in a layer of medium between slide and coverslip.

EXPERIMENTAL RESULTS

On microscopic examination of the cells in transmitted light or in the light of natural luminescence (Fig. 1a-c) both single LG and clusters of them were visible. During aging of the culture the number of LG in the cells increased, but in the case of cells cultured with $5 \cdot 10^{-4}$ M MF their number grew much more slowly. Usually at the end of the logarithmic phase and/or at the beginning of the stationary phase of growth, the number of clusters of LG in both types of culture was sufficient for micro-

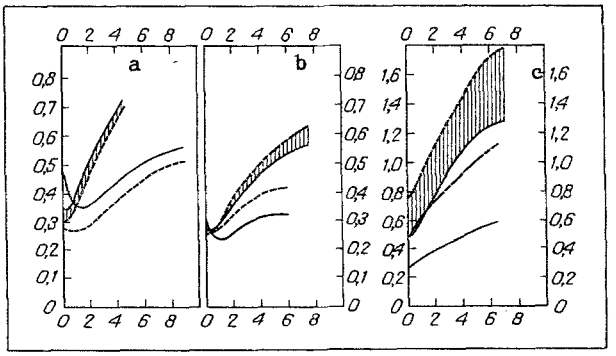


Fig. 2. Kinetics of intensity of natural luminescence of LG (a-c) from different hybridoma cells at wavelengths of 460 nm (continuous curves) and 540 nm (broken curves) on application of exciting UV, when cultured in ordinary medium and in the presence of $5 \cdot 10^{-4}$ M MF (shaded regions). a, b) Kinetics when 460-nm maximum was dominant at beginning of recording of spectra, c) 540-nm at beginning of recording of spectra. Here and in Fig. 3: abscissa, time (in min); ordinate, intensity of luminescence (in relative units).

spectrofluorometric analysis. The spectrum of natural luminescence of clusters of LG is the result of superposition of two main bands of luminescence (Fig. 1d, curve 2): in the regions of 440-460 nm (Fig. 1d, curve 1) and 540-560 nm (Fig. 1d, curve 3). Granules with a redder hue had a higher intensity of luminescence in the 600-700 nm region. Considering the composite character of the spectrum, wavelengths of 460 and 540 nm (on the slopes of the spectral curve) were used subsequently for analysis of the kinetic curves.

Kinetic curves of the intensity of natural luminescence of LG at wavelengths of 460 nm (continuous line) and 540 nm (broken line) and with continuous exposure to UV are given in Fig. 2a-c. Regardless of the initial predominance of intensity of 460 nm in the luminescence spectrum (see Fig. 2a, b) or of 540 nm (see Fig. 2c), intensities with these wavelengths initially showed quite a considerable fall. After reaching a certain minimal value the intensity of luminescence then began to rise, to flatten out on a more or less well defined plateau. Sometimes both intensities (at 460 and 540 nm) grew at almost the same rate and the shape of the common spectrum was virtually unchanged, but as a rule the intensity of 540 nm grew faster (see Fig. 2b), changing the shape of the common luminescence spectrum of LG greatly. Similar changes in the luminescence spectrum of LG were observed in my investigations on human tissues [3]. Hence it can be concluded that chromophore compounds, giving luminescence in the 540-560 nm region, are mainly responsible for changes in the spectrum of natural luminescence of LG. The character of the kinetic curves suggests that they describe the fast and slow stages of photochemical conversions of the chromophore compounds: in the fast stage the intensity of luminescence falls, in the slow stage it rises.

Culturing hybridoma cells in the presence of $5 \cdot 10^{-4}$ M MF did not change the shape of the kinetic curves (the shaded areas in Fig. 2a-c), but the rates of rise of intensity increased, and thus all the changes became clearer.

The kinetics of intensity at a wavelength of 540 nm in individual hybridoma cells grown in the presence of $5 \cdot 10^{-4}$ M MF in response to subsequent applications of UV radiation is illustrated in Fig. 3a-d. To each successive exposure to UV the duration of the fast phase of the response gradually decreased to zero (see Fig. 3a, curve 9). This took place against a background of an increase in total intensity, ultimately flattening out on a plateau. With a longer dark period (minutes) between successive applications of UV, a stage of rapid decline of the intensity of luminescence again developed; under these circumstances the depth of the minimum increased to a value higher than in response to the first excitation (compare curves 6, 8, and 10 and curve 1 in Fig. 3b). On the whole the picture leaves the impression of exhaustion of a certain compound, which is restored in darkness.

Further investigations showed that UV is only the triggering stimulus for photochemical conversions of LG. Interrupting the excitation by a time of the order of 50% of the duration of the stage did not affect the general shape of the kinetics of intensity in that stage. This kind of behavior of the natural luminescence of LG suggests the presence of "light" and "dark" photochemical conversions of chromophores in LG. The microspectrofluorometric data obtained for clusters of LG from different tissues in situ [3, 10], and the kinetic changes in natural luminescence of LG observed in hybridoma cells in vivo also suggest the existence of several fluorescent photochemical products in the 540-560 nm region.

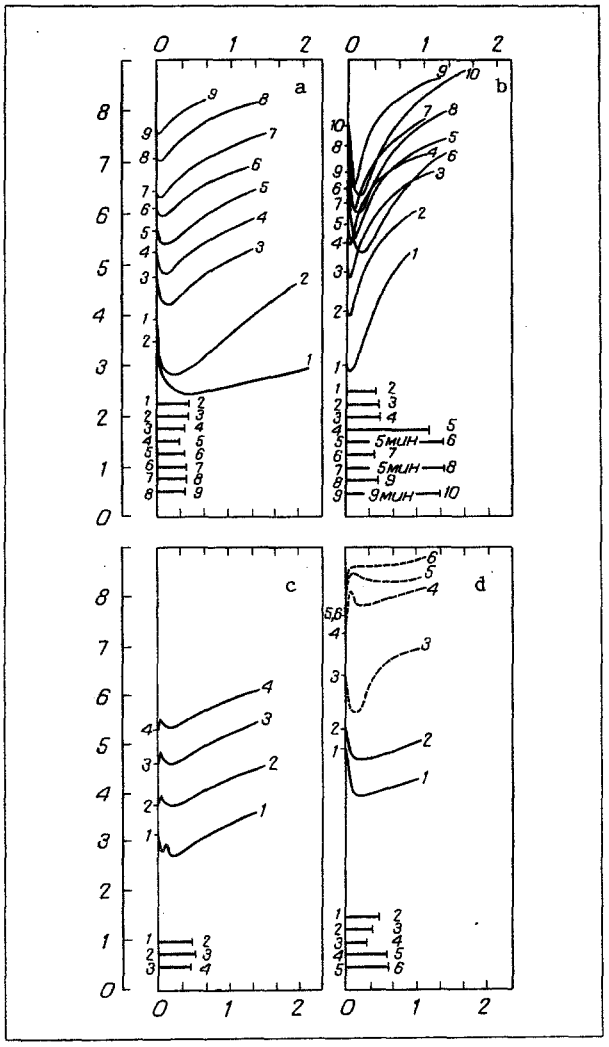


Fig. 3. Family of consecutive kinetic curves, identified by numbers, and intensities of natural luminescence of LG from different hybridoma cells (a-d) at wavelength of 540 nm on application of exciting UV and during culture of cells in the presence of $5 \cdot 10^{-4}$ M MF. d) Continuous curves (1, 2) — consecutive kinetic curves before action, and broken curves (3-6) — after action of digitonin on cell membrane. Short lines parallel to abscissa represent time of dark period between corresponding consecutive kinetic curves, identified by numbers.

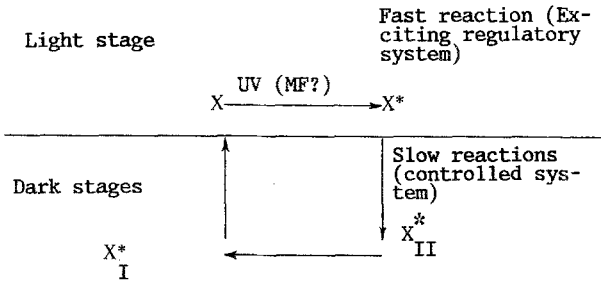
Single cells with multiple small or large protrusions of the cell membrane are sometimes observed in hybridoma cell cultures grown in the presence of MF. The kinetic curves of luminescent responses of LG in a cell with one large protrusion of the cell membrane are illustrated in Fig. 3c. An "overshoot" can be clearly seen in the fast stage of fall of intensity. Since these types of kinetics are characteristic of cells with an altered state of their cell membrane and their membrane-bound cytoskeleton, an attempt was made to modify the state of the membrane and, against this background, to study the kinetics of the luminescent responses of LG to UV. For this purpose digitonin solution was added to a drop of culture fluid, with cells, to a final concentration of 0.03%. The subsequent responses to exciting UV during the action of digitonin on the cell (Fig. 3d) revealed all the types of change of kinetic behavior described previously, namely the "deep minimum" (curve 3), in this case without a long dark period, the "overshoot" (curves 4 and 5), as on cells with an altered state of the membrane, and flattening out of the rise of intensity on a plateau (curve 6) in a much shorter time than on cells with an undisturbed plasma membrane. Thus the processes reflected in the state of the chromophore compounds in LG can be influenced by disturbing the cell membrane. More gentle

action on the plasmalemma probably may also affect the kinetics of the luminescent responses of LG. There is reason to suppose the MF may be an agent of this kind.

According to data in the literature MF is hydrolyzed in the culture medium with a half-decay time of about 1 h [5], and that the cell cycle of hybridomas lasts from 12 to 16 h. For cells of the next generation the MF concentration is already several orders of magnitude lower and is close to the concentrations of substances acting at the receptor level. Attention must again be drawn to the fact that MF modifies adenylate cyclase activity [9]. It is probably the fast, declining stage of the photochemical conversions that is connected with changes at the cell membrane level. The time of the minimum on the kinetic curves lies in the region of a tenth of a second, which is in agreement with the transmission by diffusion of a certain (probably chemical) signal from the cell membrane to LG.

The results described above suggest that MF acts on the cell membrane, possibly as a signal even at the cell receptor level, and it is in response to this that the number of LG themselves in the cells also is ultimately reduced [7].

On the basis of the results of the luminescence microspectral analysis of LG in different tissues in situ we can postulate the existence of several luminescent chemical compounds in the 540-560 nm region [3, 10] but we can describe combined kinetic curves of intensity at 540 nm in LG, depending on exciting UV, in hybridoma cells only by means of several kinetic curves, i.e., several luminescent chromophore (X) in LG described above might be described by the scheme given below:



The author is grateful to V. N. Karnaukhov, Yu. A. Mantsygin, and V. I. Popov, Senior Scientific Assistants at the Institute of Biological Physics, Academy of Sciences of the USSR, for all kinds of help with the experimental technique, and to S. I. Beilina and N. Ya. Orlov for valuable comments in the discussion of the experimental results.

LITERATURE CITED

1. V. N. Karnaukhov, V. A. Yashin, A. M. Shamarov, and V. V. Dudarev, Tsitologiya, **17**, No. 4, 478 (1975).
2. Yu. A. Mantsygin and N. V. Svyatukhina, Tsitologiya, **27**, No. 7, 822 (1985).
3. A. B. Tatariunas and V. N. Karnaukhov, Arkh. Patol., No. 9, 49 (1980).
4. A. B. Tatariunas, N. Ya. Orlov, and V. N. Karnaukhov, Byull. Éksp. Biol. Med., **103**, No. 5, 543 (1987).
5. W. Cooke, B. M. Zuckerman, and K. Schneider, Age, **2**, 13 (1979).
6. H. Miyazaki, K. Nambu, Y. Minaki, et al., Chem. Pharm. Bull., **24**, 713 (1976).
7. R. Ohtani and S. Kawashima, Exp. Gerontol., **18**, 105 (1983).
8. S. Parthasarathy, R. K. Cady, D. S. Kraushaar, et al., Lipids, **13**, 161 (1978).
9. V. Petkov, D. Zafirov, Y. Panova, et al., On the Pharmacology of Centrophenoxine, V. Petkov (ed.), Sofia (Bulgaria), (1978), pp. 28-33.
10. A. B. Tatariunas, Lipofuscin, Hungary (1987), p. 55.
11. J. Thuillier, G. Thuillier, and P. Rumf, Nature, **188**, 152 (1960).
12. C. Viticchi, S. Gentile, and L. Piantanelli, Arch. Gerontol. Geriat., **3**, 77 (1984).
13. W. G. Wood, C. Gorka, H. J. Armbrecht, et al., Life Sci., **39**, 2089 (1986).