THREE-PHASE RESPONSE OF MOCK-CELL CULTURE TO LONG-TERM ANTIVIRAL CHEMOTHERAPY

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UDC 615.281.8.015.44:611.018.15]:519.86

KEY WORDS: parameters of cell structure and function; three-phase cell response; antiviral chemotherapeutic agents; mathematical modeling

Mathematical models are now widely used in various branches of biology and medicine [7, 8], but extremely rarely in virologic practice [11]. There have been virtually no observations on changes in the structural and functional parameters of monolayer cultures during long-term exposure to antiviral chemotherapeutic agents.

The aim of this investigation was to study the action of inhibitors of viral activity on the structural and functional state of cells in a culture test system in order to construct models of the cytotoxic effect of chemotherapeutic agents.

EXPERIMENTAL METHOD

A culture of Madin–Darby canine kidney cells (MDCK) was used in the experiments. Rimantadine was obtained from the Institute of Organic Synthesis, Academy of Sciences of the Latvian SSR, and deitiforin from the Institute of Organic Chemistry, Academy of Sciences of the Ukrainian SSR. The preparations were added at the time of monolayer formation to complete growth medium containing 10% embryonic calf serum, in doses for deitiforin of 25, 50, 100, 500, and 1000 μg/ml, and for rimantadine of 25, 50, and 100 μg/ml. The maximal incubation time with the chemotherapeutic agents was 96 h. To study the effect of these agents on synthesis of macromolecules, a cell culture was grown in flasks for scintillation counting. Synthesis of the cell macromolecules was evaluated by determining incorporation of the radioactive precursors ^{14}C-valine, ^{14}C-leucine, ^{3}H-uridine, and ^{3}H-thymidine (0.4 mBq/ml in each case) into the acid-insoluble cell fraction. Labeling was carried out in medium 199 for 30 min. Morphological preparations, made from cells grown on coverslips, were stained with hematoxylin by Heidenhain's method. For the morphological and functional analysis of the cultures, a "Leitz-TAS" television analyzing system (West Germany) was used with an algorithm and programs specially developed for this purpose. The area of the nuclei was measure, their shape factor determined, and the mitotic activity of the cells estimated. The results were analyzed on the Hp-85 computer, using the method of regression analysis, and by approximation by analytical functions in order to construct mathematical models of the time course of the structural and functional characteristics.

EXPERIMENTAL RESULTS

Rimantadine and deitiforin, during the first few hours after addition to the cell culture in doses of up to 100 μg/ml for the former and 500 μg/ml for the latter caused no visible cytodestructive changes in the MDCK monolayer. Changes began to appear 12-24 h after the addition of rimantadine and 24-48 h after the addition of deitiforin. We know that the development of structural pathological or morphological changes in cells is preceded by a change in size of the nuclei [4, 6]. Karyometric analysis by the TAS system showed that both agents caused significant hypertropy of the nuclei after incubation for 24 h with the cells. Changes in size of the nuclei were accompanied by an increase in the shape factor, i.e., by a change in shape of the nuclei from ellipsoid, characteristic of intact cultures, to spherical. The nuclei of the cell population thereby became more homogeneous in shape, as

Department of Inhibitors of Viral Activity, Belorussian Research Institute of Epidemiology and Microbiology, Ministry of Health of the Belorussian SSR, Minsk. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 107, No. 5, pp. 595-598, May, 1989. Original article submitted February 23, 1987.

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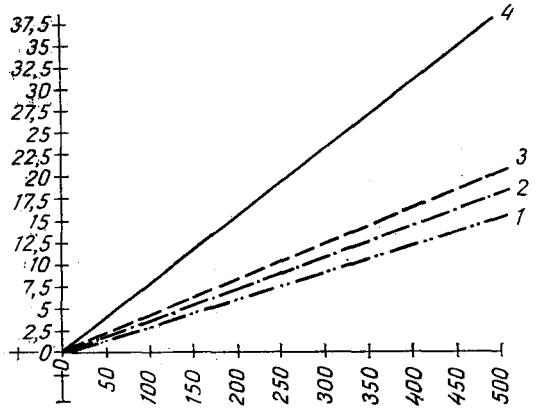

Fig. 1. Regression analysis of increase in size of nuclei of MDCK cell culture depending on dose of antiviral chemotherapeutic agents. Abscissa, dose of chemical agents (in μg/ml); ordinate, dimensions of nuclei (in μ^2). 1, 2, and 3) Deitiforin, acting for 2, 4, and 6 h respectively on cells; 4) rimantadine acting for 6 h on cells.

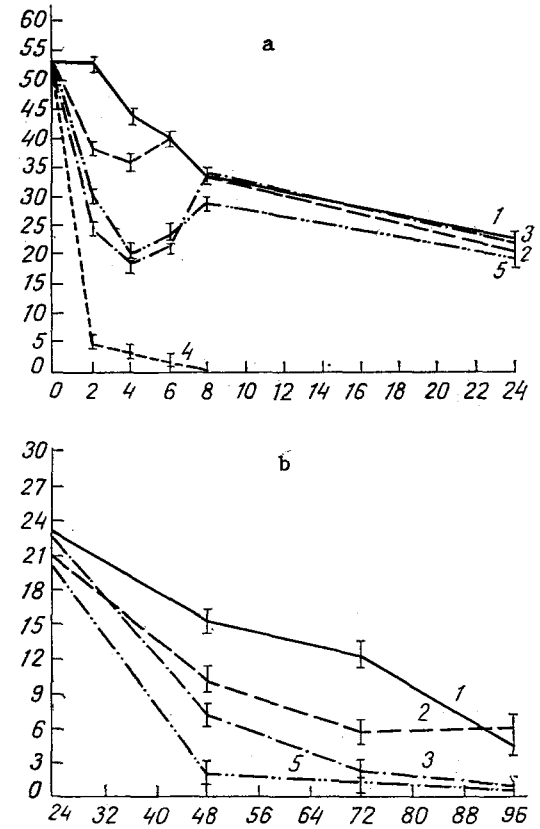

Fig. 2. Change in mitotic activity of cells of MDCK culture under the influence of antiviral chemotherapeutic agents. Changes in mitotic activity on 1st day (a) and during subsequent 3 days (b) after addition of agents. Abscissa, time (in h); ordinate, mitotic activity (in %). Here and in Fig. 3: 1) control; 2, 3, and 4) deitiforin in doses of 100, 500, and 1000 μg/ml respectively; 5) rimantadine in a dose of 100 μg/ml.

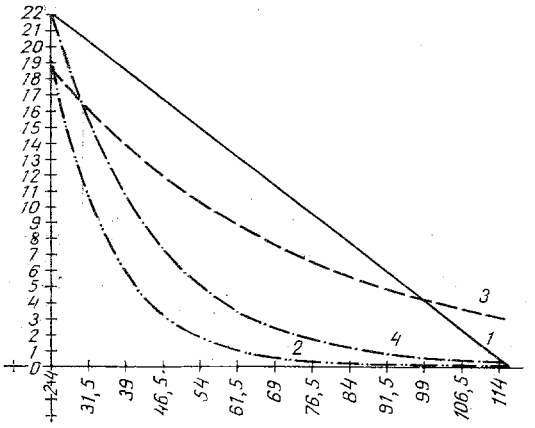

Fig. 3. Regression analysis of change in mitotic activity of cells of MDCK culture depending on duration of culture with chemotherapeutic agents (phase 3). 1) Control; 2) rimantadine 100 μg/ml; 3 and 4) deitiforin in doses of 100 and 500 μg/ml respectively.

shown by a decrease in the coefficient of variation, reflecting the scatter of values relative to the mathematical expectation. Regression analysis revealed a clear relationship between the changes in these parameters and the duration of action of the preparations and the doses added. These relationships were linear in character and described by linear first-degree equations (Fig. 1). The method of least squares was used to approximate the experimental data. The regression coefficients were calculated by the equations:

$$y = a + bx, \text{ where } b = \frac{\Sigma x_i y_i - \frac{\Sigma x_i \Sigma y_i}{n}}{\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}};$$

$$a = \left[\frac{\Sigma y_i}{n} - b\frac{\Sigma x_i}{n}\right];$$

$$r^2 = \frac{\left[\Sigma x_i y_i - \frac{\Sigma x_i \Sigma y_i}{n}\right]^2}{\left[\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}\right]\left[\Sigma y_i^2 - \frac{(\Sigma y_i)^2}{n}\right]}.$$

The accuracy of choice of the coefficients was estimated by Fisher's test.

The study of mitotic activity of the MDCK cells revealed three phases of the action of rimantadine and deitiforin on their proliferative activity. In phase 1 the mitotic index of the cells decreased in the course of 4 h after addition of the chemotherapeutic agents, and then recovered 6-8 h after their addition to the level of the intact culture. In phase 2 the mitotic activity of the MDCK cells was identical in both intact cultures and those treated with the chemotherapeutic agents (Fig. 2a). In phase 3 mitotic activity fell in the experimental cultures, starting after 24 h in vitro and continuing throughout the subsequent period of observation (Fig. 2b). The effects of the chemotherapeutic agents on proliferative processes in phase 3 are described by different regression equations. Data on the dynamics of activity under the influence of deitiforin in a dose of 100 μg/ml can be approximated by a power function. For deitiforin in a dose of 500 μg/ml and rimantadine in a dose of 100 μg/ml the character of the relationship is exponential (Fig. 3).

Coefficients of regression were calculated by the equations:

$$y = ae^{bx}, \; b = \frac{\Sigma x_i \ln y_i - \frac{1}{n}(\Sigma x_i)(\Sigma \ln y_i)}{\Sigma x_i^2 - \frac{1}{n}(\Sigma x_i)^2},$$

$$a = exp\left[\frac{\Sigma \ln y_i}{n} - b\frac{\Sigma x_i}{n}\right],$$

$$r^2 = \frac{\left[\Sigma x_i \ln y_i - \frac{1}{n}\Sigma x_i \Sigma \ln y_i\right]^2}{\left[\Sigma x_i^2 - \frac{(\Sigma x_i)^2}{n}\right]\left[\Sigma (\ln y_i)^2 - \frac{(\Sigma \ln y_i)^2}{n}\right]}.$$

Thus the quantitative microscopic study of the cytoxic action of the chemotherapeutic agents revealed a three-phase response of the cells.

The study of the time course of nucleic acid and protein synthesis in the culture of MDCK cells on the basis of incorporation of labeled precursors demonstrated the effect of the chemotherapeutic agents not only on the phase of mitosis, but also on other phases of the cell cycle, including cells in the resting phase (Table 1). These experimental data also confirmed a phasic response of the cell population. Previously, with an exposure of 24 h [1], the initial decline and subsequent recovery of levels of cellular RNA synthesis were observed in a chick embryonic fibroblast culture under the influence of rimantadine in a dose of 25 μg/ml. By using a combined technique, accompanied by mathematical methods of analysis of the experimental data, we were able to detect a regular pattern in the response of the cell population to the action of the chemotherapeutic agents. These phases can be distinguished in this response: 1) transient inhibition, 2) relative compensation, and 3) a cytopathic destructive process. Considering that a monolayer cell culture is a population of cells which obeys definite rules in its development [5, 10], it has to be pointed out that the sequence of phases which we observed is characteristic of the whole cell population, and the resultant of the responses of individual cells. Under these circumstances the responses of individual cells to an exogenous influence will evidently be determined primarily by the phase of the cell cycle in which the cells find themselves at each individual moment, whereas the response of the cell population will be determined by the integral response of the individual cells and by the particular features of intercellular interaction. Succession of phases in the response of the cell population may not necessarily be observed in cases when chemotherapeutic agents irreversibly blocking cell metabolism are used.

These data on phases in the response of the cell population may be important when the toxicity of chemotherapeutic agents is compared. Particular attention must be paid to the evaluation of the test system chosen for use in cell culture experiments. Phase 1 enables the degree and strength of the compensatory properties of the test system to be judged, on the grounds that if nontoxic concentrations are used, the dependence will be linear or weakly curvilinear, whereas if toxic doses are used, adaptation and compensation of the processes will not take place. Depending on the duration of phase 2 it is possible to judge the sensitivity of the test system: if the duration of the phase is considerably increased, such a system will not be sensitive enough and cannot be used for evaluation. Reliable results can evidently be obtained by comparing the toxic effect in phase 3 — the phase of development

TABLE 1. Time Course of DNA, RNA, and Protein Synthesis in Culture of MDCK Cells in Presence of Rimantadine (B) and Deitiformin (C), M ± m

Duration of exposure to compound, h	Radioactivity of acid-insoluble fraction, cpm								
	DMA			RNA			protein		
	A	B	C	A	B	C	A	B	C
2	2801±125	1460±67	1923±92	2453±125	2108±114	2202±30	1820±30	1573±116	1699±120
4	2751±98	1689±85	2230±87	2385±115	2168±110	2196±59	1784±45	1550±98	1692±113
6	2732±171	1871±79	2658±81	2438±75	1843±71	2048±72	1848±70	1260±75	1794±95
8	2755±153	2713±63	2794±79	2471±91	2272±80	2398±53	1815±65	1899±118	1998±65
24	2847±99	2832±51	2815±111	2406±80	2368±45	2416±75	1852±111	1856±120	1850±73
48	2551±108	1057±68	1777±85	1911±78	1143±65	1817±67	1719±90	1503±78	1468±69
72	2313±131	1110±92	1281±63	1713±110	907±25	1173±70	1607±85	1268±69	1350±125
96	2218±179	1159±43	1814±86	1685±115	529±59	859±85	1609±91	805±54	1112±58

Legend. A) Control. Dose of preparations 25 μg/ml.

of cytopathological processes. For example, the ability of the cells to take up preparations of the adamantadine group and to accumulate them in their cytoplasm was demonstrated previously. The content of these preparations inside these cells may be several times greater than their concentration in the external nutrient medium [2, 9]. Deitiformin evidently possesses similar, but weaker, ability, as shown by the exponential mathematical relationship describing the action of deitiformin in a dose of 500 μg/ml and of rimantadine in a dose of 100 μg/ml on the time course of the change in mitotic activity of MDCK cells, i.e., deitiformin is 5 times less toxic than rimantadine, in agreement with data obtained previously by other methods [3].

Thus the combination of techniques used, including methods of quantitative microscopy together with appropriate mathematical analysis of the data, revealed some general principles in the action of inhibitors of viral activity on a monolayer cell culture, and showed that their action is accompanied by a response of the cells in three phases. Depending on the duration and relative intensity of these phases it is possible to judge the strength of the cytotoxic action of agents interacting with the cells and the sensitivity of the culture as a test system and to compare the sensitivity of cells of different origin to the action of chemotherapeutic agents.

LITERATURE CITED

1. M. K. Indulen, V. A. Kalninya, G. M. Ryazantseva, and V. I. Bubovich, Mechanisms of the Antiviral Action of Adamantane Derivatives [in Russian], Riga (1981), pp. 54-55.
2. I. V. Kiseleva and N. L. Korchanova, Vopr. Virusol., No. 3, 297 (1982).
3. K. N. Kozeletskaya, Problems and Prospects in the Study of Nucleosides, Bicycloheptane, Adamantane, and Other Antiviral Compounds in Experimental and Clinical Practice [in Russian], Minsk (1982), pp. 54-56.
4. L. J. Pobrezhaite and K. V. Konstantinavicius, General Mechanims of Cell Responses to Damaging Action [in Russian], Leningrad (1977), pp. 155-156.
5. R. Baserge, Functional Regulation at the Cellular and Molecular Levels, New York (1982), pp. 3-12.
6. A. S. Belmont and C. A. Nicolini, J. Cell Sci., 58, 201 (1982).
7. K. Goerttler, Arch. Pathol., 106, No. 13, 657 (1982).
8. L. Y. Koss, Anal. Quant. Cytol., 4, No. 4, 251 (1982).
9. D. D. Richman, P. Jazahi, and K. J. Hostetler, Virology, 112, No. 1, 81 (1981).
10. J. A. Smith and L. Martin, Proc. Natl. Acad. Sci. USA, 70, 1263 (1973).
11. J. L. Taylor, W. J. O'Brien, and A. Goldman, J. Virol. Meth., 8, No. 3, 225 (1984).