Comparative Analysis of Effects of Various Types of Double-Stranded RNA on Cultured Human Laryngeal Cancer Cells

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We compared the effects of Na^+ and Ca^{2+} double-stranded RNA on cultured human laryngeal cancer cells by cytomorphometry and cytophotometry. Both agents inhibited proliferation and other cell functions, but to a different extent: Ca^{2+} double-stranded RNA was more active than Na^+ double-stranded RNA.

Key Words: *double-stranded RNA; human laryngeal cancer cell culture; nucleus; nucleolus; DNA*

Double-stranded RNA (dsRNA) and their analogs are often used as activators of metabolic processes in different cell populations [2]. Na^{2+} dsRNA, an inductor of interferon, possesses also immunomodulating and antiviral activities and stimulates metabolism and regeneration [2,7]. The effects of Ca^{2+} dsRNA on cells are less studied, but its antitumor effects was reported [1] and, according to our data [6], injection of Ca^{2+} dsRNA to mice with experimental anemia stimulated erythropoiesis and specialization of erythroid cells. Even this brief review shows differences in the effects of Na^+ and Ca^{2+} dsRNA on cells, which can be due to the presence of different ions and to the fact that experiments were performed on different objects. This problem could be solved by performing experiments on the same cell population and comparison of identical parameters, which became the aim of our study: analysis of the effects of Na^+ and Ca^{2+} dsRNA on cultured human laryngeal cancer cells (Hep-2). The study of the effects of these compounds on transformed cancer cells will also provide more reliable information about their antitumor activity.

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MATERIALS AND METHODS

RNA with a molecular weight of $20\text{-}150\times10^3$ Da was isolated from total yeast RNA by fractionation, concentration, and purification and tested for double helixes [1,11]. Then Ca^{2+} dsRNA was obtained by the method of Graham and van der Eb [10]. Na^+ dsRNA was received from Kirchenstein Institute of Microbiology, Latvian Academy of Sciences. Hep-2 culture was received from the bank of D. I. Ivanovskii Institute of Virology, Russian Academy of Sciences.

Two-day suspension culture of Hep-2 cells was transferred into penicillin flasks with slides and cultured in Eagle-MEM medium with glutamine and 10% bovine serum until the formation of a monolayer in the absence or presence of either Na^+ dsRNA, or Ca^{2+} dsRNA. The agents were added in a concentration of 10 μg/ml (this concentration was determined in studies of the concentration-effect relationship for Na^+ dsRNA [3]). Proliferative activity of cells, behavior of their nuclei and nucleoli, the dynamics of their size, and DNA content were studied by cytomorphometry and

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cytophotometry. The slides were removed after 24, 48, and 72 h. The preparations were stained with hematoxylin (by the method of Hansen) and eosin after fixation in 96% ethanol. For DNA detection the preparations were stained with fuchsin (by the method of Feulgen) after fixation in a mixture of ethanol and acetic acid (3:1) with 2% formalin and hydrolysis in 5 N HCl (22°C, 60 min) [5].

The density of cell monolayer (number of cells per unit area), number of nucleoli in the nuclei, and the mitotic coefficient (for 1000 cells) were determined. The weight of DNA in the nuclei and perinuclear chromatin (PNC), area and perimeter of each nucleus and nucleolus were measured by TV scanning (100/1.30 objective; λ=575 nm) using an image analysis system created in our Department on the base of SMP-05 photometer microscope (Opton) and UPIAM-2000 software. Before scanning images of the nuclei and nucleoli (including PNC) were contoured with an "optic pen". The areas and perimeters of the nuclei and nucleoli were used for estimating their volumes ($V=4/3\pi R^3$) and surface areas ($P=4\pi R^2$). A total of 100 cells were analyzed in each case. The data were expressed in arbitrary units.

Along with arithmetic means, weighed means were estimated for each group [8]. The significance of differences between the means was evaluated using Student's *t* test.

RESULTS

The data presented in Table 1 suggest that the density of cell monolayer in the control was high (14.7±0.2 cells/unit area) and virtually did not change during 3 days of culturing. In the presence of Na^+ dsRNA this parameter was 3-fold lower (to 3.7±0.6 cells/unit area). In contrast, in the presence of Ca^{2+} dsRNA the density of cell monolayer increased almost 2-fold during the same period and was 10.9±0.1. This parameter depends on the intensity of cell multiplication and death, which increased during culturing in both control and experimental cultures, especially in cultures treated with Ca^{2+} dsRNA. Proliferative activity was high (though differed significantly) in all groups. For example, the mitotic coefficient was 4.03% in the control, 2.47% after addition of Ca^{2+} dsRNA, and 2.25% in the presence of Na^+ dsRNA. This indicates different, but always inhibitory effects of dsRNA on cell proliferation ($p<0.001$). Table 1 shows that the mean number of nucleoli decreased in the presence of both dsRNA. This is associated with redistribution of nuclear population by the number of nucleoli towards nuclei with lesser number of the nucleoli (Fig. 1). Simple estimation shows that the percentage of mononucleolar nuclei after 3 days of culturing was 15.3% in the control, 25.6% after treatment with Na^+ dsRNA, and 30% after treatment with Ca^{2+} dsRNA. The percentage of binucleolar nuclei increased from 30% in the control to 42% after addition of Na^+ dsRNA and to 33.6% after addition of Ca^{2+} dsRNA.

The percentage of multinucleolar (3-5) nuclei decreased from 18.1% in the control to 16.8 and 13.6% after addition of Na^+ dsRNA and Ca^{2+} dsRNA, respectively. If we regard the presence of many nucleoli as an indicator of functional activity, these data indicate its suppression primarily under the effect of Ca^{2+} dsRNA, which is in line with decreased content of DNA in PNC zones under the effect of Na^+ dsRNA and more

TABLE 1. Monolayer Density, Mitotic Activity, Number of Destroyed Cells, and Mean Number of Nucleoli in the Nuclei of Intact Hep-2 Cell Culture (Control) and after Treatment with Na^+ dsRNA and Ca^{2+} dsRNA

Group	Duration of culturing, h	Number of cells per unit area ($\bar{x}\pm S_{\bar{x}}$)	Mitoses, %	Destroyed cells, %	Mean number of nucleoli ($\bar{x}\pm S_{\bar{x}}$)
Control	24	13.7±0.5	2.6	4.0	2.4±0.2
	48	15.1±0.4	3.5	3.3	2.8±0.2
	72	15.2±0.5	6.0	1.6	2.8±0.2
		14.7±0.2*			2.73±0.07*
Na^+ dsRNA	24	3.4±0.1	2.5	5.9	2.1±0.1
	48	4.0±0.2	2.0	5.0	2.4±0.2
	72	4.2±0.6	1.1	2.6	2.8±0.2
		3.7±0.6*			2.35±0.05*
Ca^{2+} dsRNA	24	8.7±0.2	2.8	8.8	2.1±0.2
	48	11.5±0.3	2.5	6.1	2.1±0.2
	72	15.5±0.5	2.1	4.6	2.5±0.3
		10.9±0.1*			2.21±0.08*

Note. Here and in Table 2: *weighed means.

TABLE 2. DNA Content (Arb. Units), Volume (V), and Surface Area (P) of Nuclei and Nucleoli during Growth of Intact Hep-2 Cells and in Cultures Treated with Na^+ and Ca^{2+} dsRNA ($x \pm \bar{S}_x$)

Group	Duration of culturing, h	Nucleus			Nucleolus		
		DNA	V	P	DNA (perinuclear chromatin)	V	P
Control	24	103.9±4.7	238.6±11.1	82.6±2.4	20.6±1.1	17.5±0.6	20.9±1.2
	48	105.9±4.7	258.0±6.8	85.7±1.6	20.2±2.2	21.1±1.3	27.0±1.2
	72	160.2±8.8	421.4±18.3	150.7±2.2	36.8±3.3	36.8±3.0	35.1±2.4
		116.0±2.0*	285.0±4.2*	106.5±0.7*	23.0±0.6*	20.6±0.4*	27.3±0.5*
Na^+ dsRNA	24	106.7±3.8	356.4±11.7	106.2±3.1	16.3±0.7	18.3±0.6	19.1±1.2
	48	112.0±4.5	369.6±20.0	110.2±2.6	26.8±2.2	26.8±1.9	25.2±2.0
	72	157.6±9.5	443.2±28.3	133.8±4.0	27.8±2.8	31.6±3.2	34.1±3.2
		117.0±1.7*	379.4±5.6*	116.1±1.0*	19.7±0.4*	21.8±0.4*	24.1±0.6*
Ca^{2+} dsRNA	24	117.6±5.5	336.3±14.3	99.9±1.2	16.1±0.9	19.1±1.1	14.3±0.8
	48	122.3±6.6	346.7±14.7	105.5±1.6	16.2±0.9	17.5±0.8	17.4±1.2
	72	124.9±8.8	369.3±17.4	111.4±1.6	22.5±2.1	17.4±0.4	17.9±1.1
		121.0±2.0*	351.5±5.0*	105.0±0.5*	17.3±0.4*	17.6±0.2*	16.2±0.3*

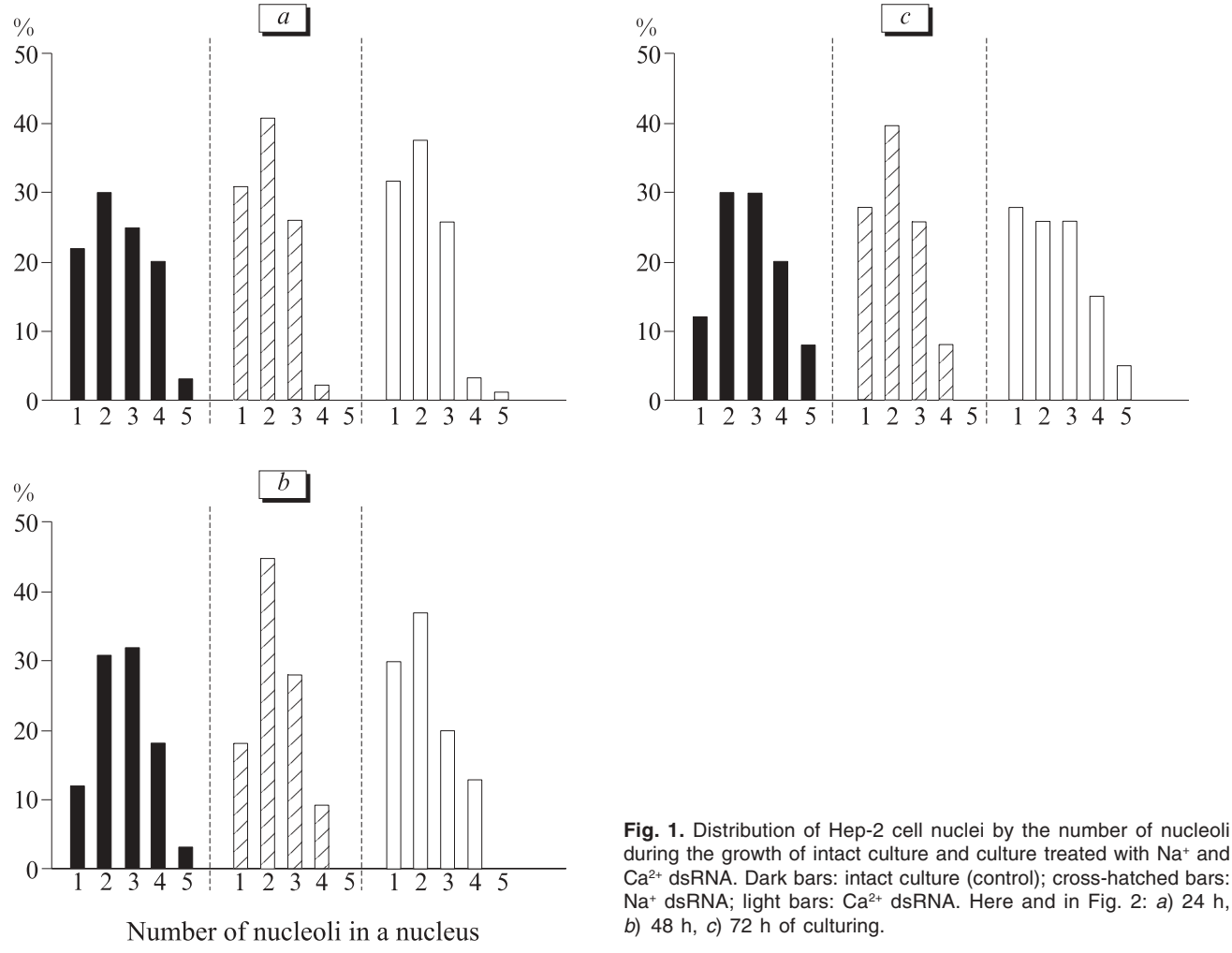

Fig. 1. Distribution of Hep-2 cell nuclei by the number of nucleoli during the growth of intact culture and culture treated with Na^+ and Ca^{2+} dsRNA. Dark bars: intact culture (control); cross-hatched bars: Na^+ dsRNA; light bars: Ca^{2+} dsRNA. Here and in Fig. 2: *a*) 24 h, *b*) 48 h, *c*) 72 h of culturing.

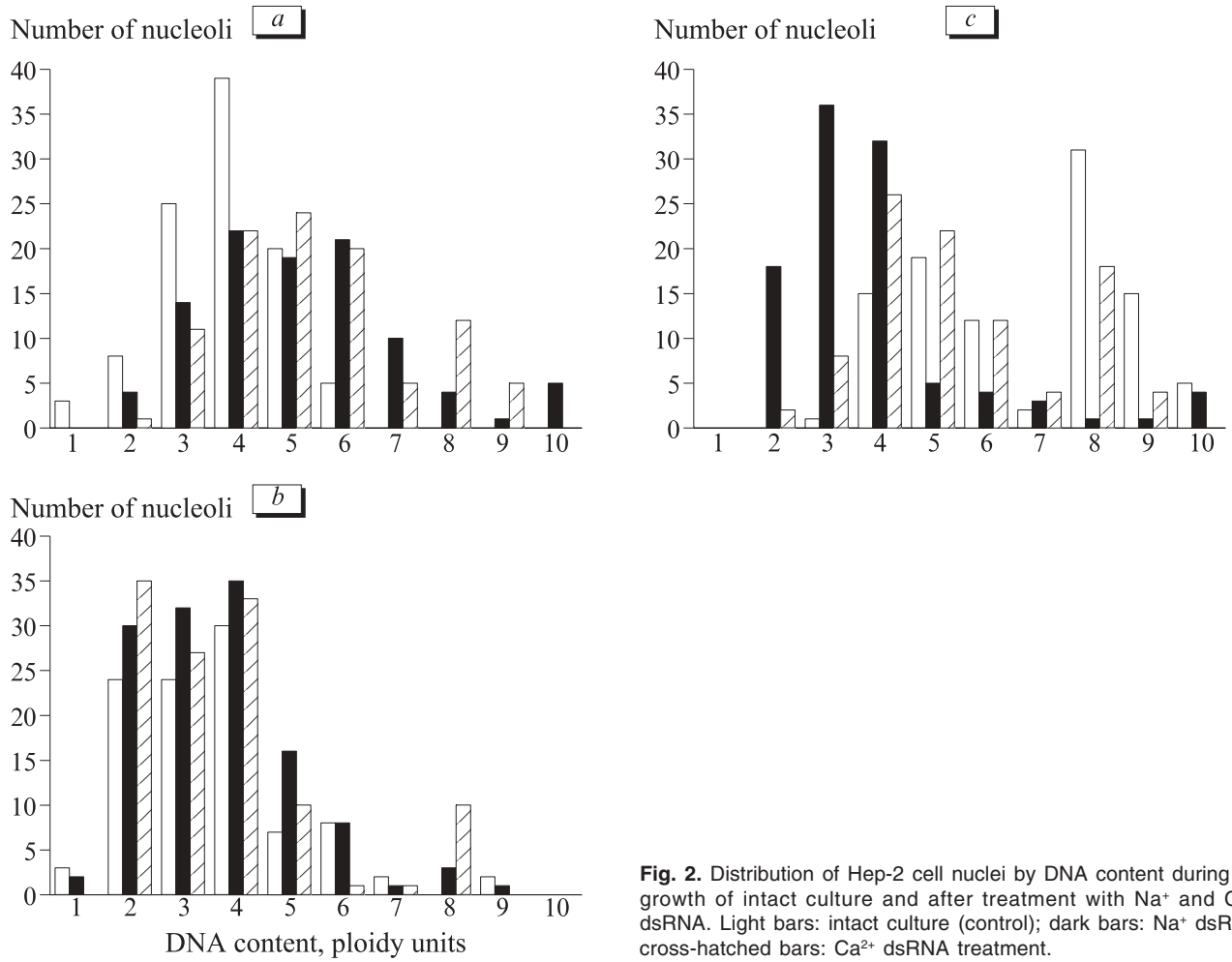

Fig. 2. Distribution of Hep-2 cell nuclei by DNA content during the growth of intact culture and after treatment with Na^+ and Ca^{2+} dsRNA. Light bars: intact culture (control); dark bars: Na^+ dsRNA; cross-hatched bars: Ca^{2+} dsRNA treatment.

so Ca^{2+} dsRNA (Table 2). Table 2 shows that addition of these compounds induced no appreciable changes in the mean content of DNA in the nuclei, volumes and surface area of the nuclei in comparison with the control, but culture growth was accompanied by redistribution of the nuclei by their ploidy (Fig. 2).

In the control the percentage of 2*c*-4*c* cells was maximum after 24 and 48 h of culturing; after 72 h 2*c* cells disappeared, the percentage of 4*c* cells decreased, while the number of 8*c* cells increased. In cultures treated with dsRNA the percentage of 2*c*-4*c* cells after 24-h culturing was below the control, and numerous 4*c*-8*c* cells appeared. After 48 h the percentage of 2*c*-4*c* cells increased and that of 8*c* cells decreased. After 72 h the distribution in cultures with Na^+ dsRNA was the same as after 48 h, while in cultures treated with Ca^{2+} dsRNA the distribution shifted towards high-ploidy cells.

In parallel, numerous nuclei with "intermediate" DNA content were detected in all groups. This phenomenon is usually regarded as aneuploidy, but is not always associated with it. The values between 2*c* and 4*c* were most likely caused by cell passage through *S* phase of the cell cycle or even their lagging in the second half of this phase [9,14]. It is also possible that the appearance of 4*c*-8*c* cells is a result of blocking in the G_2 phase or passage through the *S* phase of the next cycle without completion of division in the previous cycle, which is seen from the presence of 8*c* cells in the population. Arrest in the G_2 phase and formation of polyploid cells in parallel with decreased proliferative activity are usually attributed to cell recruitment into the differentiation processes [4]. Detection of hypodiploid cells after 24 and 48 h in culture can be explained by the development of nucleolysis in dying cells, rather when aneuploidy, particularly if we take into consideration the fact that hypodiploid cells die after 72 h. However, the object of our study were transformed cells, and therefore we cannot rule out the possibility of aneuploidy, particularly remembering that variability in the number of chromosomes in these cultured cells with a mode of 77 chromosomes was previously reported [8,13].

Our findings confirm the inhibitory effects of tested dsRNA derivatives on proliferation and other vital activities of cultured Hep-2 cell and demonstrate vari-

ability of these shifts. It is noteworthy that addition of $CaCl_2$ into the culture medium and particularly NaCl in preliminary experiments induced no significant changes in Hep-2 cells. Addition of "pure" dsRNA just nonspecifically stimulates cell growth according to published reports [2].

Thus our experiments and previous studies demonstrated a specific effect of dsRNA in complex with Na or Ca ions on cells. The data on the use of dsRNA and their analogs for regulation of metabolic processes (mainly in immune cells) [10], attempts at explanation of the molecular mechanisms of their activity [12], and our present findings indicate that the presence of dsRNA, though not modulating the pattern of intracellular metabolism in principle, stimulates or inhibits the intrinsic functions of the cells. Despite scanty information about many components in the chain of molecular events involved in the regulation of proliferation and differentiation under the effect of dsRNA, we hypothesize with good grounds that this should be paralleled by activation of intracellular membrane enzymes (cyclases) mediating accumulation of cAMP and cGMP in cells. These latter activate a series of cAMP- and cGMP-dependent protein kinases which, in turn, regulate important metabolic processes determining, as we showed, at the cellular level the type of growth, multiplication, and differentiation. We therefore consider that the key initialing point triggering the entire chain of events resultant from the treatment with agents used in our study is an increase in intracellular concentration of cyclic nucleotides.

From medical viewpoint, the most important result of this study is that both Na^+ and especially Ca^{2+} dsRNA suppress multiplication and stimulate the polyploid transformation of cells, thus decreasing the level of population malignancy in general. The latter fact creates prerequisites for trying these low-molecular-weight compounds under conditions of malignant growth *in vivo* with the aim of their prospective use in medical practice.

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