METHODS

A Comparative Analysis of Radiometric and Autoradiographic Methods for studies of DNA-Synthesizing Activities of Cells *In Vitro*

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The DNA synthesis in cultured Ehrlich ascites carcinoma cells was studied by autoradiography and liquid-scintillation radiometry. The kinetics of total radioactivity of incorporated ^{3}H-thymidine correlated with the kinetics of the index of radioactivity. The radiometric assay provided an extremely precise estimation of changes in the DNA synthesis *in vitro*. In several cases the autoradiographic assay was more sensitive. We assumed that autoradiography and liquid-scintillation radiometry are interchangeable methods for studies of DNA synthesis *in vitro*. These assays are recommended for studying the effects of cell proliferation inhibitors on DNA synthesis.

Key Words: *autoradiography; Ehrlich ascites tumor; liquid-scintillation radiometry; proliferation inhibitors; DNA synthesis*

In modern experimental biology, radiometric methods are more frequently used than the traditional autoradiographic assay [1-3,8,11,12] because autoradiography is an extremely laborious assay. Radiometry is a simple and rapid procedure. Radiometric methods provide integrative information on DNA synthesis in cells. However, the cell entry into the S phase of mitotic cycle and the synthesis of DNA during the S phase cannot be studied individually. Autoradiography allows one to determine the relative number of DNA-synthesizing cells (the index of radioactivity, IR) and the intensity of DNA synthesis in cells (the labeling index of nuclei) [5]. Sometimes, only one of these indices is analyzed during the autoradiographic assay. This decreases the informativeness of autoradiography [7, 13,14]. However, precise autoradiographic assays considering both the IR and the labeling index of nuclei, characterized cell transitions during the mitotic cycle [4,9,10]. These indices are important for analysis of the effects of inhibitors and stimulators of cell proliferation that specifically affect the mitotic cycle phases and DNA synthesis.

A chalone-containing preparation (CCP) from Ehrlich ascites tumor (EAT) was shown to contain low-molecular-weight peptides inhibiting proliferation [3]. Here we analyzed and compared the informativeness of autoradiography and liquid-scintillation radiometry regarding the DNA synthesis in cultured EAT cells using CCP.

MATERIALS AND METHODS

Diploid strain of EAT cells (Institute of Chemical Physics, Russian Academy of Sciences) was maintained in BALB/c mice by periodical reinoculations.

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CCP was obtained from abdominal tumor cells of mice at the thirteenth day after inoculation of the tumor [6].

Medium 199 containing 2 mM L-glutamine and 10% fetal bovine serum was used for culturing of tumor cells *in vitro*. Reagents for cell structures were from Sigma. ^{3}H-Thymidine (1.5 μCi/ml) was added to the culture 1 h before sampling for analysis of the DNA synthesis by liquid-scintillation radiometry. DNA was precipitated on nitrocellulose filters with trichloroacetic acid. The filters were air-dried and analyzed in a Rack-beta liquid-scintillation counter (LKB) using a toluene scintillator.

^{3}H-Thymidine (30 ml) with a specific activity of 0.01 μCi/ml was added into 1 h before sampling autoradiography. Each histological preparations was covered with type M photoemulsion (NIIKhIMPhOTOproekt), which was heated to 40°C and diluted with distilled water (1:1). The preparations were dried at room temperature and exposed at 4°C in the dark. The exposition time was 7 days. The preparations were developed with a methyl-hydroquinone developer, fixed with 30% sodium thiosulfate, and stained with methylene blue. The relative number of DNA-synthesizing cells (IR) and the mean number of silver grains over 50 nuclei (labeling index of nuclei) were then determined. The cell was considered to be labeled if there were seven and more silver grains over it. Not less than 5000 cells were examined in each preparation.

Data were analyzed statistically using Student's *t* test. The differences were considered as statistically significant at $p<0.05$.

Table 1. Total Index of Radioactivity (x10^3) in Cultured EAT Cells after the Addition of CCP (*M±m*)

Time after CCP addition, h	Control	Experiment	Inhibition, %
2	44.6±1.7	28.8±0.7	35.4
3	53.0±3.1	22.3±0.8	57.9
4	61.8±3.8	7.4±0.6	88.0
5	35.3±1.4	0.9±0.3	97.5
6	25.6±4.0	0±0	100

Note. All results differ significantly from control ($p<0.001$)

Table 2. IR in Cultured EAT Cells (‰) after the Addition of CCP (*M±m*)

Time after CCP addition, h	Control	Experiment	Inhibition, %
2	326.0±14.3	310.7±17.9	—
3	323.0±12.7	335.3±8.2	—
4	311.3±16.0	223.0±27.1*	28.4
5	276.7±13.6	23.3±7.3**	91.6
6	267.0±11.6	15.3±5.2**	94.3

Note. Here and in Table 3: *$p<0.05$ and **$p<0.001$, respectively, compared with control.

Table 3. Labeling Index of Nuclei in Cultured EAT Cells after the Addition of CCP (*M±m*)

Time after CCP addition, h	Control	Experiment	Inhibition, %
2	26.3±2.4	25.0±1.7	—
3	33.0±2.7	28.0±2.3	—
4	21.7±2.7	14.3±0.9*	34.1
5	23.0±3.8	8.3±1.2*	63.9
6	15.7±3.0	7.3±0.7*	53.5

RESULTS

Table 1 shows the effects of CCP on DNA synthesis in cultured EAT cells (liquid-scintillation radiometry). The total index of radioactivity reflecting the radioactive tritium decay in 1 million cells over 1 min was estimated. CCP decreased the total index of radioactivity in the EAT culture starting from the second hour of observations. Kinetic analysis of this parameter showed that CCP-induced inhibition of DNA synthesis in EAT cells increased during further culturing. The maximum decrease in the total index of radioactivity under the effect of CCP was observed after 5 and 6 h. By the 6th h this parameter was practically undetectable in the experimental series. Therefore, DNA was not synthesized in the EAT culture. The relatively high confidence levels ($p<0.001$) of the differences between all experimental and control series (Table 1) was the advantage of liquid-scintillation radiometry.

The IR decreased significantly by the 4th h after the addition of CCP. This effect of CCP then increased (Table 2). It can be suggested that CCP delayed the S phase of EAT cells. The maximum inhibition of the IR was also observed 5 and 6 h after the addition of CCP. The IR was inhibited by 94.3%. Autoradiography showed that 15.3±5.2‰ of EAT cells continued to synthesize DNA the 6th of the experiment. The dynamics of the increase in IR inhibition after the 4th h corresponded to the dynamics of the inhibition of the total index of radioactivity.

Table 3 shows CCP-induced changes of the labeling index of nuclei in cultured EAT cells. Statistically significant inhibition of this parameter was observed 4 h after the addition of CCP (similar to IR inhibition). The maximum inhibitory effect of CCP was observed on the 5th h of experiment. The effect slightly de-

creased on the 6th h (63.9% inhibition after 5 h and 53.5% inhibition after 6 h). Thus, CCP inhibits DNA synthesis in the S phase. A comparative analysis of Tables 2 and 3 showed that the inhibition of DNA synthesis was less pronounced than the delay of the S phase. This is probably due to the fact that the sensitivity of cells in the G_1-S phase of mitotic cycle to CCP is higher than that of cells in the S phase.

The total index of radioactivity was similar to the IR (Tables 1-3). This is probably due to the fact that CCP predominantly delayed the S phase of EAT cells, which contributed to a decrease in total radioactivity.

The priority and efficiency of methods used for measuring the DNA-synthesizing activity are important for studies of biological effects of inhibitors of cell proliferation. Our autoradiographic assay showed that the DNA synthesis in cultured EAT cells is regulated at two levels: the entry of cells into the S phase and their transformations during the S phase. Autoradiography showed that the DNA synthesis in EAT culture is retained. However, the entry of cells into the phase of synthesis decreased by 94.3%, and the intensity of DNA synthesis in the S phase decreased twofold. Liquid-scintillation radiometry did not detect DNA-synthesizing activity of cultured EAT cells at this stage of experiments. This was probably due to extremely low contribution of the IR and labeling index of nuclei into the total radioactivity during this period. Thus, autoradiography provided a more precise assessment of DNA-synthesizing activity of EAT cells at late stages of our experiments *in vitro*.

By contrast, on the 2nd and 3rd h more precise results were obtained by liquid-scintillation radiometry. The inhibitory effect of CCP was detected by this method, while the autoradiographic assay showed no significant changes. Obviously, there was an extremely small decrease in the IR and labeling index of nuclei at the early stages of experiment. Therefore, only the total index of radioactivity which depends on DNA synthesis in the S phase and the number of cells in the S phase can change significantly. It should be emphasized that the results of radiometry were more reliable than those of autoradiography. This was probably due to extremely precise measurements of radioactivity by radiometry because DNA of numerous EAT cells (3 millions tumor cells in each experimental point) was analyzed on a liquid-scintillation counter.

Our results show that autoradiography and liquid-scintillation radiometry are complementary methods for studies of DNA-synthesizing activities of cells *in vitro*. A combined use of these assays would be appropriate for analysis of the effects of cell proliferation inhibitors on the DNA synthesis.

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