THE DETECTION OF DEAD AND LIVING CELLS BY THE METHOD OF FLUORESCENT MICROSCOPY

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The problem of differentiation of dead and living cells is a very important one in various biological fields. For this reason the development of methods to do this deserves special attention. As long ago as 1940, Strugger [20] and also Bukatsch and Haitinger [11] suggested using for this purpose the fluorochrome stain acridine orange (AO), which they had produced. Strugger developed a test of viability in cells of plants, bacteria and yeasts. From his results he concluded that living cells fluoresce a green color and dead cells red, with transitional forms orange. Strugger's findings were confirmed in investigations by other workers [13-17, 19, 21] on various biological objects.

At the same time, the test proposed by Strugger was subjected to severe criticism in papers by many other workers who concluded that this method cannot be used to determine the viability of cells [2, 4-6, 9, 10, 12, 18]. G. A. Medvedeva [3], in M.N. Meisel's laboratory, proposed the use of the acid fluorochrome dye primulin in distinguishing living and dead cells of yeasts.

In order to ascertain the suitability of acridine orange and primulin for the detection of living and dead animal cells, we carried out experiments with ascitic cells from an Ehrlich's tumor exposed to various inactivating agents. The work was done in conformity with all the conditions of fluorochrome technique as given in the paper by Vinegar [21], who concluded that AO could be used for determining the viability of animal cells.

EXPERIMENTAL METHOD

All the experiments using inactivating agents and the control experiments were carried out with one sample of ascitic fluid.

This fluid was first diluted 1:100 with phosphate buffer (pH = 6), after which the concentration of cells in such a suspension was determined in a Goryaev counting chamber. This concentration was found to be 75 $\times 10^6$ cells per cm^3. Phosphate buffer was then added to the suspension in a volume calculated to give a suspension containing 10^5 cells per cm^3. The suspension obtained was poured into tubes and used for the experiments with the inactivating agents. The following series of experiments were carried out.

1. Control series. 1 cm^3 of the suspension was inoculated (i.e. 10^5 cells) without additional treatment.

2. Inactivation by high temperature. Before inoculation, the tube with the suspension was placed on a water bath at a temperature of 60° for 3 minutes.

3. Irradiation with x-rays. Irradiation was with an RUM-3 apparatus at a temperature of 0° and with the following technical conditions: voltage 180 kv, current 10 ma, focal distance 30 cm, filters 0.5 Cu + 1.0 Al; total dose 3000 r.

Relative Number (in per cent) of Tumor Cells of Various Types of Fluorescence after Fluorochrome Staining and the Successful Innoculation of a Cell Suspension After Treatment with Various Agents

Experimental series	Successful inoculation of ascitic carcinoma*	Mean time of death of mice from ascitic carcinoma in days	Acridine orange			Primulin + AO		
			I	II	III	I	II	III
			solitary red granules or absence of granules	large number of red granules or diffusely pink cytoplasm	cytoplasm full of large granules of diffusely red	very faint dull green fluorescence of cytoplasm	yellowish pink cytoplasm	yellow-green fluorescence of the whole cell
1. Control (with 100,000 tumor cells)	20/20	8.9 ± 0.3	30.0	40.5	29.5	50.4	48.0	1.6
2. Inactivation by high temperature	0/10	–	18.0	63.0	19.0	8.0	10.0	82.0
3. Irradiation with x-rays	0/10	–	19.2	56.8	25.0	25.0	11.6	80.3
4. Inactivation with alcohol	0/10	–	16.0	60.2	23.8	1.0	6.6	82.4
5. Autolysis at 37° for 10 hours	4/10	38 ± 5.1	23.2	51.6	25.2	23.0	19.0	58.0
6. Autolysis at 37° for 24 hours	0/10	–	21.8	52.2	26.0	25.0	14.0	61.0
7. Inactivation by sarcolysin for 10 hours	9/10	18.4 ± 1.2	13.8	61.2	25.0	38.0	50.0	12.0
8. Inactivation with sarcolysin for 24 hours	7/10	27.7 ± 0.8	11.7	63.0	25.3	21.0	33.0	46.0
9. Control of the experiments with sarcolysin and autolysis	10/10	11.6 ± 0.5	28.0	42.8	29.2	54.0	44.0	2.0

* The denominator is the number of mice in the experiment, the numerator the number of mice in which an ascitic carcinoma developed.

4. Inactivation with alcohol. The suspension was mixed with 30° alcohol in a proportion of 1:1 and incubated for 10 minutes at +4°, then centrifuged for 2 minutes at 1000 rpm, after which the precipitated cells were resuspended in phosphate buffer.

5-6. Autolysis. The suspension was incubated at 37° for 10 or 24 hours.

7-8. Inactivation by sarcolysin. A solution of sarcolysin in phosphate buffer (100γ/ml) was mixed with the suspension of ascitic cells in a proportion of 1:1. The mixture was incubated for 10 or 24 hours, after which it was centrifuged and the precipitated cells were resuspended in phosphate buffer.

9. Control series to the experiments with autolysis and sarcolysin. The suspension was incubated for 24 hours at 4°.

After the inactivating agents had been applied, 2 ml of suspension was taken from each tube for examination under the fluorescent microscope. Fluorochrome staining with AO was carried out, 1 ml of a 2×10^{-4} solution of AO being added to 1 ml of the cell suspension. A study of the fluorescent microscopic picture of the ascitic cells, stained with AO, enabled them to be divided into 3 groups according to the nature of fluorescence of their cytoplasm: 1) the cytoplasm was a dull green color with solitary red granules; 2) the cytoplasm was diffusely pink in color, or had a large number of red granules; 3) the cytoplasm was diffusely red or filled with very red granules.

On fluorochrome staining with primulin, the living ascitic cells did not show up. For this reason, to reveal the outlines of the cells (nucleus and cytoplasm) we added to the primulin solution (1:10,000) a few drops of AO in the same concentration. To 1 ml of cell suspension 1 ml of 2×10^{-4} primulin was added. By this method of fluorochrome staining the ascitic cells were divided into 3 groups according to the nature of their fluorescence: I, dull green cytoplasm, green nucleus; II, yellowish pink cytoplasm, green nucleus; III, yellowish green fluorescence of the whole cell (nucleus and cytoplasm difficult to distinguish).

During all the cell counts the relative number of cells of each group in 1000 tumor cells was determined. The material from each experimental series was inoculated intraperitoneally into 10 mice; all the experimental mice received the same volume of suspension (1 ml) as the control mice (see above). The mice were observed for 2 months after inoculation.

EXPERIMENTAL RESULTS

The results of the experiments are shown in the Table.

It must be pointed out that we judged the degree of inactivation of the ascitic tumor cells both by the decrease in the percentage of successful inoculations of the tumor and by the changes in the time of death of the mice from ascitic carcinoma. We know from the literature [1, 21 and others] that both of these indices are functions of the number of viable ascitic carcinoma cells injected. As may be seen from the table, in the control series (series 1 and 9) the ascitic carcinoma took successfully in all the animals and death ensued after a short time (in the second week of the experiment). The inactivating agents used could conventionally be divided into 2 categories by the degree of inhibition of growth of the Ehrlich's carcinoma. The "weak" inactivating agents included those which lowered the percentage of successful inoculations of the tumor and retarded its growth. These were autolysis at 37° for 10 hours (series 5) and incubation of the tumor cells mixed with sarcolysin for 10 or 24 hours (series 7 and 8). The "strong" inactivating agents were those which caused the percentage of successful inoculations of a suspension of ascitic cells to fall to zero. These were high temperature (series 2), irradiation by x-rays (series 3), the action of alcohol (series 4) and autolysis at 37° for 24 hours (series 6).

Let us compare the results of the experiments in which suspensions of ascitic cells were inoculated after the application of various agents and the results of the counts of the relative numbers of cells with the various types of fluorescence after fluorochrome staining of the same suspensions with AO. The only more or less regular difference between the control and experimental series was a slight increase in the number of group II cells (with a large number of granules or diffusely pink cytoplasm) and a fall in the number of group I cells after the use of the inactivating agents. This increase, however, was not very significant (roughly from 40 to 50-60%) and was not proportional to the degree of inactivation of the ascitic cells. The relative number of group III cells in general was hardly changed.

We did not therefore observe that marked alteration in the fluorescence of the cells which might have been expected from Strugger's ideas, i.e. the appearance of a red fluorescence of the cytoplasm instead of green in response to the inactivating agents. Both normally and after inactivation tumor cells with differing amounts of fluorescent cytoplasmic substance could be seen in the suspension. Our experiments agreed well with the conclusions of M. N. Meisel' and B. V. Korchagina [7] and of certain other workers [8, 9], who showed that this red fluorescence was characteristic of structures containing ribonucleic acid. Some experiments which we performed in fact showed that by the action of crystalline ribonuclease (1 mg enzyme to 1 ml of distilled water; incubation for 1 hour at 37°) on films of ascitic carcinoma, previously fixed with Carnoy's fluid and stained with fluorochrome AO, a substance with a red fluorescence completely disappeared from the cytoplasm of the tumor cells. Hence it could be concluded that the changes which we mentioned above in the relative content of cells with various types of fluorescence evidently reflect changes caused by the inactivating agents in the ribonucleic acid content of the cytoplasm of the ascitic cells. As may be seen from the above, examination of the fluorescence of cells stained with AO cannot be used as a method of detecting living and dead cells in a suspension, although it is a very valuable method of study of the nucleic acids of the cell during life.

Different results are given by an analysis of the data of examination of the fluorescence of the same cell suspensions after fluorochrome staining with primulin. Particularly demonstrative changes were found here in the relative number of group III cells, intensively combining with the fluorochrome, and hence giving an intense yellow-green fluorescence of the whole body of the cell. In the control series these cells did not account for more than 1-2% of all the cells; after the use of "weak" inactivating agents the number of group III cells rose to 12-58%. Under these circumstances in the series of experiments in which the degree of inactivation of the tumor cells was least (after incubation with sarcolysin for 10 hours; series 7) the group III cells were only 12% of the total number. At the same time, when the length of incubation was increased to 24 hours (series 8), and the inactivation became more marked, the percentage of these cells rose to 46. After autolysis for 10 hours (series 5), causing still more marked inactivation of the cells, the number of group III cells reached 58%. Finally, in all the experiments using "strong" inactivating agents (series 2, 3, 4, 6) the group III cells accounted for 61-82% of the cells in the suspension.

Thus the percentage of cells intensively accumulating primulin changes in parallel with the degree of inactivation of the ascitic carcinoma cells. There are, therefore, grounds for considering that the cells of this category are inactivated. It is difficult to determine the character of the group II cells with their weak yellowish pink fluorescence of the cytoplasm. There are many such cells in the ascitic cell suspensions of both experimental and control series; these cells may possibly be completely viable, but we cannot yet be sure that among them a variable number of injured cells may not be present. It follows from the above that examination by the fluorescent microscope of cells stained with primulin can be used as a rough guide to the determination of the relative number of living and dead cells in cell suspensions. It is not yet clear at what stage of injury to the cell the accumulation of fluorochrome begins. The problem of whether only cells which have irreversibly lost their viability may be detected in this way therefore requires further study.

SUMMARY

Results of fluorescent microscopic investigation of suspension of Ehrlich ascitic carcinoma cells treated in vitro with different inactivating agents were compared with results of biological experiments by which the viability of these cells has been tested. It was concluded that the examination of the fluorescence of cells stained with acridine orange cannot be used for differentiation of the living from the dead cells. At the same time this technique may serve as a valuable method for supravital examination of both nucleic acids. Examination of the fluorescence of cells stained with primuline can be used for the approximate estimation of the percentage of viable cells in the cell suspensions.

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