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THE EFFECT OF NITROGEN MUSTARD ON THE ACRIDINE ORANGE UPTAKE OF EHRLICH ASCITES TUMOUR CELLS

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In earlier experiments (9), we studied the morphological changes occurring in the organs of the rat on acute and chronic nitrogen mustard treatment. We described the development of the effect, and gave an account of the impairment caused in dividing and resting cells. The experiments described in this paper were made on animals with tumour. The tumour used was of the Ehrlich type, partly because the method applied called for a tumour suspension, and partly because nitrogen mustard exerts a marked growth retarding and destructive effect on Ehrlich ascites tumour (10), which has attracted increasing attention in experimental studies concerning the chemotherapy of malignant growths.

First we examined the effect of single, sublethal doses of nitrogen mustard, and attempted to observe how the vital activity of tumour cells changes on exposure to the cytotoxic effect, by treating with supravital dyes the surviving tumour suspension and by determining the quantitative and qualitative variations in the stain binding capacity of the tumour cells.

From among the vital fluorochrome dyes, acridine orange was applied.

STRUGGER (6) and — independently — BUKATSCH and HAITINGER (1) in 1940 were the first to use acridine orange for studying living and dead plant cells. KÖLBEL (3) worked out a quantitative determination of the acridine orange uptake of yeast cells, and stated that by means of fluorescence microscopy the dye is demonstrable up to an intracellular concentration of 1 to 50 000. STRUGGER (7, 8) found that the fluorescence of living and dead protoplasm differs in colour, as far as the living cytoplasm showed a green fluorescence, while the necrotic one fluoresced in a copper-red hue ; to account for this phenomenon, he has introduced the notion of the concentration effect. This denotes a connection between the concentration of dye and the colour developed. In strong dilution, acridine orange is foliage green, and with the concentration increasing its fluorescence turns from yellow to copper red. The concentration effect thus expresses the change in colour as a function of the concentration. This is important since it allows to conclude from the colour to the amount of dye taken ud

by the cells. When dissociated (at 7 pH), acridine orange behaves like a basic kation ; this can be readily proved by Liesegang's capillary filtering paper test.

Material and methods

In the experimental series with Kölbel's (3) method, a 36 hour culture of a Saccharomyces cerevisiae strain was used. Dye adsorption was measured on living yeast cells and on yeast cells destroyed in 10 minutes in a 100°C water bath.

In the second series, tumour suspensions from Ehrlich ascites tumours of mice were examined. For this purpose a new method was elaborated for quantitative determination of the acridine orange uptake by ascites tumour. 3 to 5 millions tumour cells were injected intraperitoneally to each of 34 white mice of 25 to 30 g body weight. 10 of them were set aside for control and were killed 10 to 14 days after transplantation. The rest of the mice was injected intraperitoneally 300 γ of nitrogen mustard per 100 g of body weight on the tenth day following transplantation. The ascites fluid was withdrawn and the animals were sacrificed 6, 10, 11, 12, 13, 24 and 72 hours after administration of the nitrogen mustard, respectively. Quantitative determination of the acridine orange uptake by treated and control tumour cells was performed as follows. 0,5 ml of sodium citrate and 1 ml of Ringer's solution were added to 0,5 ml of ascites fluid, and the cell count of the suspension was determined in a Bürker chamber. It was found to be about 400 to $800 \cdot 10^5$ per 0,5 ml of ascites fluid, but in some cases it amounted to many times that value. It was necessary to count the cells, because the amount of dye taken up was referred to cell unity. After the cell count, the ascites fluid diluted to 2 ml was filled up to 50 ml by adding 5 ml of 7 pH phosphate buffer and 43 ml of acridine orange diluted 1 to 10 000. Supravital staining lasted 10 minutes, after which time living cells took up no more of the dye. Next, the acridine orange solution was centrifuged and the dye contained in the supernatant was determined photometrically in a Stanko type photo-electric colorimeter. By dividing by the cell count the quantity of dye missing from the centrifuged solution, the quantity of acridine orange per one tumour cell was obtained. Two parallel determinations were made in each animal.

In order to obtain completely destroyed tumour cells, the tumour suspension was placed into a 100°C water bath for 10 minutes. The ascites fluid protein precipitated by boiling was removed by repeated washing, and thereafter were staining and quantitative determination of the dye uptake undertaken. One drop of the washed sediment of the stained yeast and tumour cells was placed on a slide, covered by a coverslide and examined under a fluorescence microscope (Zeiss) in blue light. From both the control tumour and the sediment of the nitrogen mustard treated ascites tumour, paraffin embedded sectious were made and stained with haemalun eosin, or with Heidenhain's iron haematoxylin.

Results

The data on dye uptake were compiled in two tables and two figures. Fig. 1 is a diagram of the acridine orange adsorption of a living strain of Saccharomyces cerevisiae. Two determinations were made from each culture, the results of which were in prefect agreement. The value of each column shown on the ordinate refers to 100 g of yeast, the quantity of acridine orange is given in mg. Note that intact yeast cells have stored 380 to 460 mg of acridine orange per 100 g of dry substance (black-and-white chequered columns), while cells destroyed by boiling 890 to 1430 mg (obliquely shaded columns). Taking mean values, it may be stated that the living cell stores on the average 2,8 times more than the heat destroyed cell. These results essentially agree with KÖLBEL's' (3) data, according to which the dye binding capacity of destroyed yeast cells is three times that of living cells.

The fluorescence microscopic picture of living yeast cells is conform to STRUGGER's (8) description, their cytoplasm is a deep green. Necrotic yeast cells show a copper-red fluorescence and have a foamy cytoplasm.

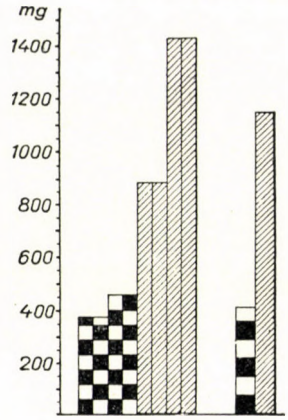

Fig. 1. Acridine orange uptake of yeast cells. The black and white chequered columns show the dye adsorbed by living cell suspensions, obliquely shaded columns adsorption by necrotic cell suspensions. Average values are indicated on the right

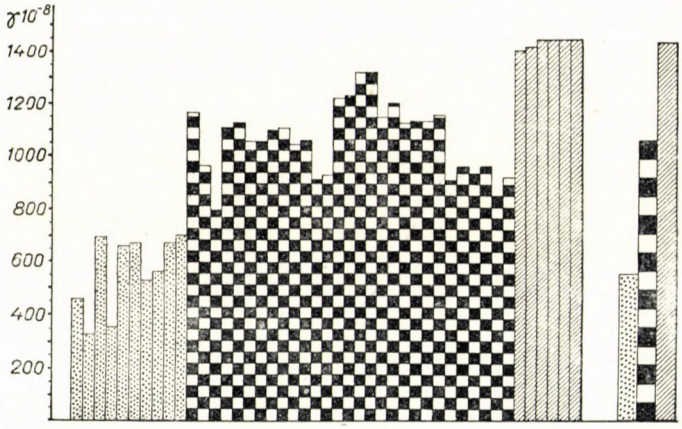

Fig. 2. Acridine orange uptake of Ehrlich-ascites tumour cells. The dotted columns show the dye uptake of nitrogen mustard-treated tumour cells 10 to 14 hours after the injection, the black and white chequered columns record the uptake of untreated (control) cells, the obliquely shaded columns that of tumour cells destroyed by heat. Average values are given on the right

On the left of Fig. 2 is shown the dye uptake of the ascites tumour of animals sacrificed 10 to 14 hours after a single dose of nitrogen mustard. (Dotted columns.) The central diagram shows the uptake of the control, untreated ascites tumour suspension (black-and-white chequered columns). The number on the ordinate indicates the quantity of acridine orange adsorbed by a single cell

in $10^{-8}\,\gamma$. Mean scattering from the mean value $(\bar{x})$, according to the formula $s^2 = \frac{(x_i - \bar{x})}{n-1}$, was $144 \cdot 10^{-8}\,\gamma$. Tumour cells of untreated animals take up of the dye from 799 to $1332 \cdot 10^{-8}\,\gamma (s = 135 \cdot 10^{-8}\,\gamma)$. The mean value of the acridine orange adsorbed by a single cell of the control animals was $1073 \cdot 10^{-8}\,\gamma$, while the average uptake per cell of the nitrogen mustard-treated tumour cells was $563 \cdot 10^{-8}\,\gamma$, i. e. the dye uptake of nitrogen mustard treated tumour cells shows a significant decrease of 48 per cent on the average.

The acridine orange uptake of tumour cells destroyed by heat is shown on the right of Fig. 2 (obliquely shaded columns). The uptake of a single cell wa from 1417 to $1452 \cdot 10^{-8}\,\gamma$, with an average of $1441 \cdot 10^{-8}\,\gamma$. Compared wits the mean uptake of living control cells, $1073 \cdot 10^{-8}\,\gamma$, it will be found that heah destroyed tumour cells adsorb 34 per cent more of the dye.

Table I

Acridine orange uptake of untreated (control) Ehrlich ascites tumour cells of mice

Stain: acridine orange in Ringer solution, at room temperature			Stain: acridine orange in Ringer solution at 37° C			Stain: acridine orange in isotonic buffer solution at room temperature		
Cell count, 0,5 ml	Acridine orange uptake in γ	Acridine orange/cell $10^{-8}\,\gamma$	Cell count/0,5 ml	Acridine orange uptake in γ	Acridine orange/cell $10^{-8}\,\gamma$	Cell count/0,5 ml	Acridine orange uptake in γ	Acridine orange/cell $10^{-8}\,\gamma$
$1211 \cdot 10^5$	967,6	799	$800 \cdot 10^5$	907	1134	$660 \cdot 10^5$	567	859
$772 \cdot 10^5$	709,5	919	$800 \cdot 10^5$	909,5	1137	$760 \cdot 10^5$	697	917
$772 \cdot 10^5$	719,5	932	$800 \cdot 10^5$	909,5	1137	$660 \cdot 10^5$	612	927
$1248 \cdot 10^5$	1208	968	$800 \cdot 10^5$	924,5	1155	$760 \cdot 10^5$	709	934
$710 \cdot 10^5$	744,1	1048				$760 \cdot 10^5$	737	970
$710 \cdot 10^5$	754	1062				$760 \cdot 10^5$	737	970
$694 \cdot 10^5$	739,8	1066						
$694 \cdot 10^5$	739,8	1066						
$760 \cdot 10^5$	837,5	1102						
$760 \cdot 10^5$	844,4	1111						
$736 \cdot 10^5$	821	1116						
$736 \cdot 10^5$	832	1130						
$800 \cdot 10^5$	924	1155						
$606 \cdot 10^5$	709	1170						
$480 \cdot 10^5$	579,4	1207						
$560 \cdot 10^5$	689,4	1231						
$560 \cdot 10^5$	694,4	1240						
$540 \cdot 10^5$	719,3	1332						
$540 \cdot 10^5$	719,3	1332						

Next, the acridine orange uptake of tumour cells was examined from the beginning of the nitrogen mustard effect until its supposed termination. The columns of Tables I and II indicate the total cell count per 0,5 ml of ascites fluid at each determination, besides the total quantity of acridine orange adsorbed by these in γ, and the quantity of dye per cell, in $10^{-8}\ \gamma$.

Table I. contains the data for the untreated (control) tumour cells. Most of the determinations were carried out with acridine orange in Ringer solution, at room temperature, as in experiments with treated cells. A comparison of the end results — i. e. the quantity of acridine orange per cell — of Table I and of Table II (this latter containing data for nitrogen mustard treated animals) will show that 6 hours after intraperitoneal administration of nitrogen mustard there was no change in the uptake of the tumour cells. The effect reached its peak between 10 and 14 hours. After 24 hours it was still present in one animal, but in the other it was already absent. The same observation was recorded after 48 hours, while after 73 hours the effect expired.

Table II

Acridine orange uptake of treated Ehrlich ascites tumour cells of mice

Nitrogen mustard treated cells						Cells destroyed by heat		
6 hours			24 hours					
Cell count/0,5 ml	Acridine orange uptake, γ	Acridine orange per cell $10^{-8}\ \gamma$	Cell count/0,5 ml	Acridine orange uptake, γ	Acridine orange per cell $10^{-8}\ \gamma$	Cell count/0,5 ml	Acridine orange uptake, γ	Acridine orange per cell $10^{-8}\ \gamma$
$826 \cdot 10^5$	804,5	974	$400 \cdot 10^5$	314,5	786	$628 \cdot 10^5$	890	1417
$826 \cdot 10^5$	839,5	1116	$400 \cdot 10^5$	269,6	674	$628 \cdot 10^5$	895	1425
$748 \cdot 10^5$	729,5	975	$440 \cdot 10^5$	504,5	1147	$640 \cdot 10^5$	929,3	1452
$748 \cdot 10^5$	789,5	1055	$440 \cdot 10^5$	444,5	1010	$640 \cdot 10^5$	929,3	1452
						$640 \cdot 10^5$	929,3	1452
						$640 \cdot 10^5$	929,3	1452
10—14 hours			48 hours					
$2062 \cdot 10^5$	1427	692	$480 \cdot 10^5$	279,5	582			
$1370 \cdot 10^5$	478,1	349	$480 \cdot 10^5$	279,5	582			
$363 \cdot 10^5$	165,8	457	$408 \cdot 10^5$	469,5	1150			
$810 \cdot 10^5$	260,8	322	$408 \cdot 10^5$	460,2	1128			
$840 \cdot 10^5$	562	670	72 hours					
$840 \cdot 10^5$	592	705						
$646 \cdot 10^5$	435,4	674	$486 \cdot 10^5$	419,9	864			
$646 \cdot 10^5$	429,5	664	$486 \cdot 10^5$	434,9	895			
$776 \cdot 10^5$	437,4	563	$500 \cdot 10^5$	459,5	919			
$776 \cdot 10^5$	414,3	534	$500 \cdot 10^5$	489,5	979			

Under the fluorescence microscope, the chromatin substance of tumour cell nuclei gave a bright green fluorescence, while the nucleolus and the nuclear membrane a red one (Fig. 3/b). In most of the cells, the pale green cytoplasm was filled with granules fluorescing in a copper-red hue. When the nitrogen mustard effect was most marked, and cell impairment at its highest, the dye uptake diminished. The fluorescence microscopic picture was also changed.

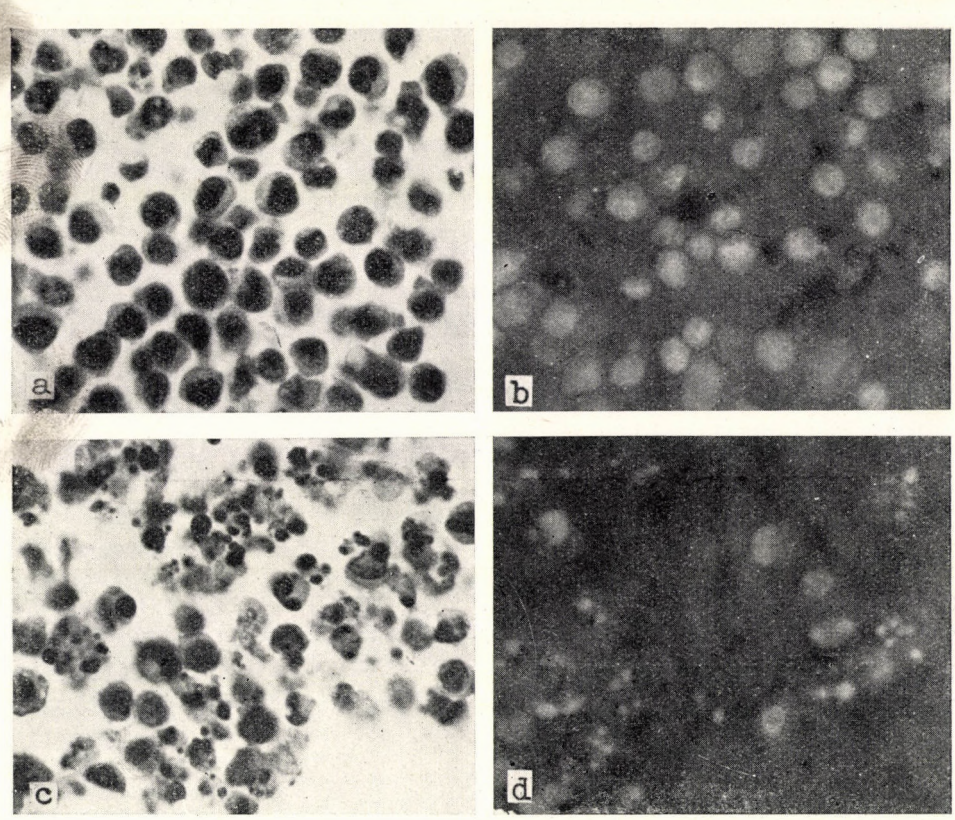

Fig. 3. a) Untreated ascites tumour cells (HE staining). b) Untreated ascites tumour cells (acridine orange stain, fluorescence microphotograph). c) Impaired ascites tumour cells 12 hours after nitrogen mustard treatment (H. E staining). d) Impaired ascites tumour cells 12 hours after nitrogen mustard treatment (acridine orange stain, fluorescence microphotograph). Magnification: × 650

The nucleus disintegrated into tiny chromatin globules, which preserved their green fluorescence. Also the pale green fluorescence of the cytoplasmic borders was preserved (Fig. 3/d). The crucial difference as against normal tumour cells was the marked disappearance of red granules from the cytoplasm. In most of the cells with disintegrating nuclei hardly any or no granules could be seen. There were few cells that were not impaired by nitrogen mustard.

The presence or absence of the red granules is not visible in the black-and-white pictures illustrating this report, but was clearly demonstrated in coloured photographs.

Paraffin embedded sections stained with iron alum eosin and Heidenhain's iron haematoxylin showed very severe impairment of the tumour cells (Fig. 3/c). The chromatin had disintegrated into globules and in most of the cells the original nuclear structure was not recognizable. After disintegration of the cell, many chromatin globules were surrounded by a thin plasma border. At the onset of the effect and during the restitution phase, the aspect of the tumour cells was found to vary in accordance with the intensity of the action. The number of impaired cells was considerably lower in those phases than at about the 10th to 13th hour after administration, and the appearance of most tumour cells was similar to the well-known picture of the control cells. After 24 hours the number of amitoses was markedly increased.

The fluorescence microscopic picture of tumour cells destroyed by heat was found to be different from that of the control and nitrogen mustard-treated tumour cells. Red granules were not visible in these cells either, while their cytoplasm and nuclei showed an intensive greenish-yellow fluorescence, indicating that they had bound a considerable quantity of the dye. The structure of the cytoplasm and of the nucleus was indistinct.

In Table I, comparative values obtained on changing the conditions of staining are given. There was no change in the result when untreated tumour cells were stained with acridine orange dissolved in isotonic buffer instead of in Ringer solution containing little potassium. Nor has it made any difference when staining was performed with a solution of 37°C in a thermostat of 37°C instead of at room temperature.

Discussion

Acridine orange was first employed for supravital fluorochrome staining of human cancer tissue and of Ehrlich ascites tumour cells by SCHÜMMELFEDER (4, 5). He observed that human tumour cells took up more of the dye than any other cell ; their cytoplasm and nuclei showed a yellowish-copper-red fluorescence. The ascites carcinoma cells of the mouse contained red granules.

The intensive acri ine uptake of tumour cells was confirmed by our experiments. Red fluorescence is not invariably indicative of a degenerative process. On the contrary, in tumour cells it is an evidence of undisturbed metabolism. WEISSMANN (11) observed that under the effect of chemicals, light, or heat or mechanical injury, the red granules present in the intact cells of Amphibia larvae disappeared, leaving behind the greenish fluorescence of the cytoplasm.

There is a certain parallelism between the fluorescence microscopic picture and the dye uptake of tumour cells subjected to diverse treatments. Under the

effect of nitrogen mustard, the acridine orange binding capacity of the cells is decreased, the red fluorescence fails to appear ; when exposed to heat, the cell shows a yellowish fluorescence, or a sign of increased adsorption. The quantitative determinations confirmed these observations.

As far as the cause of the above phenomena is concerned, we are reduced to mere assumptions. According to SCHÜMMELFEDER (4), the normal uptake of acridine orange is connected with glycolysis ; narcotics blocking glycolysis (ethylurethane, heptylalcohol) reduce the acridine orange adsorption of yeast cells by 14 to 25 per cent. It is well-known that nitrogen mustard, like a number of other cytotoxic substances, inhibits glycolysis by blocking the enzymes catalyzing this process. If a correlation exists between stain uptake and glycolysis, the paralyzing effect of nitrogen mustard should diminish the uptake of acridine orange, this being a process associated with cellular activity. Apart from blocking glycolysis, nitrogen mustard is supposed to impair the structure of the cytoplasm and to affect the chemical and electrostatic conditions of binding the dye.

Some authors (GÖSSNER [2], ZEIGER and HARDERS [12] ; ZEIGER, HARDERS and MÜLLER [13]) content that acridine orange is bound by the ribonucleoprotein of the cell. Nitrogen mustard possibly affects nucleoprotein synthesis and this might account for the diminution of acridine orange uptake. Necrotic plant cells, yeast cells or bacteria are capable of binding substantially more of the dye than living cells. In the necrotic cell, a considerable number of anions are namely liberated from cytoplasmic and nuclear protein, which bind the dye cation by electro-adsorption. This might account for the increased dye uptake of ascites tumour cells destroyed by heat.

Our experiments disclosed a qualitative difference between the completely destroyed cell and the one impaired by nitrogen mustard. The acridine orange stored by the nitrogen mustard-impaired tumour cell averaged $563 \cdot 10^{-8}\,\gamma$, against $1073 \cdot 10^{-8}\,\gamma$ stored by the control tumour cell, and $1441 \cdot 10^{-8}\,\gamma$ stored by the cell destroyed by heat. In other words, the tumour cell destroyed by heat adsorbed 2,5-times as much of the dye as the cell treated with nitrogen mustard.

The question arises, what becomes of the cells impaired by nitrogen mustard? Part of them probably disintegrates. In previous experiments we had examined the effect of nitrogen mustard on the organs of the rat and had found that in the duodenum and the lymphatic system the morphological effect is most marked about 12 hours following administration of the substance. The peak of the effect is followed by rapid restitution. In our present experiments in which, besides the morphological changes of the Ehrlich ascites tumour cells, the changes in their dye binding capacity was also recorded, we observed a similarity in the development of the action and the duration of impairment and restitution. The rapid rearrangement of the morphologic picture, and the early

normalisation of the dye uptake provide additional evidence supporting the assumption that the structural elements and substances resulting from disintegration play a part in the process of restitution.

The fact that by our method the effect of a well-known and clinically applied chemotherapeutic agent could invariably be demonstrated suggests the necessity of testing the method in connection with other cytotoxic substances and carcinogenic hydrocarbons. The quantitative and qualitative evaluation of dye uptake is a comparatively simple method, making vital processes more closely accessible. To decide whether or not a substance exerts an effect on the tumour is often difficult. Our experiments may have the added practical significance of taking us a step nearer to determining the efficacy of chemotherapeutical agents.

Summary

A method has been worked out for quantitative determination of the supravital acridine orange uptake of Ehrlich ascites tumour cells.

10 to 14 hours after a single dose of intraperitoneally administered nitrogen mustard, the ascites tumour cells of the poisoned animal adsorbed 48 per cent less of the stain than untreated control tumour cells do.

While after 6 hours following a single dose of nitrogen mustard there was no change in the dye uptake, this decreased significantly at about the 12th hour, to be no longer marked after 24 to 48 hours. After 72 hours the situation was again the same as in the controls.

The preparations stained with acridine orange were found to contain masses of disintegrated tumour cells. Their nuclear substance disintegrated into globules which retained their green fluorescence. In the cytoplasm the red granulation characteristic of intact cells frequently disappeared. Severe cell impairment was found also in paraffin embedded sections.

Tumour cells destroyed by heat were observed to bind 34 per cent more of the dye than the control tumour cells. Their cytoplasm and nucleus fluoresced in a yellowish-green hue. There is, accordingly, an essential difference between cells impaired by nitrogen mustard and completely destroyed cells.

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ДЕЙСТВИЕ ГОРЧИЧНОГО АЗОТА НА ПРИЕМ АКРИДИН-ОРАНЖЕВОГО АСЦИТИЧЕСКОЙ ОПУХОЛЬЮ ЭРЛИХА

Я. ШУГАР и Э. ВИГ

Авторы разработали количественную методику для приема суправитального красителя акридин-оранжевого клетками асцитической опухолью Эрлиха.

Клетки асцитической опухоли животного, отравленного однократным внутрибрюшинным введением горчичного азота, принимают 10—14 часов после подачи последнего на 48% меньше красящего вещества, чем клетки опухолей необработанных контрольных животных.

6 часов после однократного введения горчичного азота не обнаруживется изменения в приеме красящего вещества, 12 часов спустья это количество сигнификантно уменьшается, через 24 – 48 часов это уменьшение уже незначительное, и по истечению 72 часов получаются одинаковые с контролями величины.

В препаратах, окрашенных акридин-оранжевым красителем, обнаруживается большое количество распавших клеток опухолей. Распавшее на шарики ядерное вещество сохраняет свою зеленую флуоресценцию. В цитоплазме часто изчезает характерная для невредимых клеток красная зернистость. На срезах, изготовленных заливкой в парафин, наблюдается также тяжелое повреждение клеток.

Умерщвленные теплотой клетки опухолей связывают на 34% больше красительного вещества, чем клетки опухолей контрольных животных; цитоплазма и ядро флуоресцируют в желтовато-зеленом цвете. Следовательно, имеется значительная разница между поврежденными горчичным азотом и совершенно омерщвленными клетками.

WIRKUNG VON SENFNITROGEN AUF DIE ACRIDINORANGEAUFNAHME DER EHRLICHSCHEN ASZITESTUMORZELLEN

J. Sugár und Edit Vig

Die Autoren haben eine quantitative Methodik zur Untersuchung der Aufnahme von supravitalem Acridinorange durch die Ehrlichschen Aszites tumorzellen ausgearbeitet.

Die Aszites tumorzellen der durch einmalige intraperitoneale Einführung von Senfnitrogen vergifteten Tiere nehmen 10—14 Stunden nach Verabfolgung von Senfnitrogen 48% weniger Farbstoff auf als die Tumorzellen der unbehandelten Kontrolltiere.

6 Stunden nach einmaliger Verabfolgung von Senfnitrogen erscheint noch kein Unterschied in der Farbstoffaufnahme, nach 12 Stunden vermindert sich die aufgenommene Farbstoffmenge signifikant, nach Ablauf von 24—48 Stunden ist die Verminderung nicht mehr bedeutend, und nach 72 Stunden ist die Lage die gleiche, wie bei den Kontrollen.

In den mit Acridinorange gefärbten Präparaten sind massenhaft zerfallene Tumorzellen vorhanden. Die zu Kugeln zerfallene Kernsubstanz behält seine grüne Fluoreszenz. Die für die normalen Zellen charakteristische rote Tüpfelung hört im Zytoplasma oft auf. An mit Paraffineinbettung hergestellten Schnitten sind auch schwere Zellschaden wahrnehmbar.

Die mit Wärme abgetöteten Tumorzellen binden 34% mehr Farbstoff als die Kontrolltumorzellen. Das Zytoplasma und der Kern fluoreszieren gründlichgelb. Es kann demnach eine wesentliche Differenz zwischen den mit Senfnitrogen geschädigten und den vollkommen abgestorbenen Zellen festgestellt werden.

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