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Effects of the cytostatic drug 1-(2-hydroxyethyl)-3-(2-chloroethyl)-3-nitrosourea (HECNU) on heat production and intermediary metabolism of diploid human fibroblasts IMR 90.

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Abstract

Cellular thermogenesis was continously monitored over 72 hours by using microcalorimetry in human lung fibroblasts (IMR 90) growing in monolayer, with and without addition of HECNU in increasing concentrations of 1, 10, 100 and 500 μg/ml medium. To facilitate interpretation of the heat production data, pH, the consumption of oxygen, glucose and the production of lactate as measured in the medium as well as the content of intracellular ATP were determined in parallel cultures.

The cytostatic effect of HECNU treatment was measured as the population doubling which became reduced with 10 μg/ml HECNU or more. In control cultures HP decreased from 48 pW/cell to 20 pW/cell at completion of the experiments. This decrease in HP correlates well to the observed increase in cell number. In contrast, treatment with HECNU did not alter HP per cell number. Similarly O_2- and glucose-consumption, pH, and intracellular ATP-content apparently remained unaffected.

The results may suggest that the cytotoxicity of HECNU is not due to an influence on energy metabolism but tentatively affecting nucleic acid metabolism via the alkylating effect of the drug.

1. INTRODUCTION

Improved awareness and ethical concerns in animal experimentation necessitate the search for alternative methods. In this respect various cell culture techniques are suggested as suitable in vitro measures in assessing cellular handling of pharmaceutical products. Microcalorimetry represents a simple method to assess cellular metabolic activities [1-2] and direct measurement of heat production (HP) may thus allow conclusions about the net cellular energy metabolism. The method has been frequently emploid for the investigation of thermogenesis in cells treated with drugs [3-8] including also antineoplastic substances [4,7].

In the present work we studied the influence of the cytostatic drug 1-(2-hydroxyethyl)-3-(2-chloroethyl)-3-nitrosourea (HECNU) in increasing concentrations on the heat production of diploid human lung fibroblasts, growing in monolayer.

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2. METHODS

2.1. Cells and culture conditions

Diploid human embryonal lung fibroblasts, line IMR 90 [9], were purchased from N.I.A. Cell Culture Repository, Institute for Medical Research, Camden, New Jersey, USA. We used cells with a cumulative population doubling of 26-30. Cells were grown in Dulbecco's MEM (Gibco/BRL, Eggenstein FRG) containing 1 g glucose/l, supplemented with 10% foetal calf serum (Biochrom, Berlin, FRG), 20 mM hepes and 15 mg achromycine/l (Lederle, München, FRG). Cell numbers in duplicate cultures were determined at appropriate times in a Fuchs-Rosenthal-hemocytometer. The viability of the cells was determined by the trypane-blue exclusion method.

Cells were grown as monolayers in glass bottles under a gas phase of 95% air/5% CO_2 and incubated at 37°C. Cells of dense monolayers were suspended by trypsinization and transfered into 3 ml glass ampoules (Machery & Nagel, Düren, FRG) as described previously [8]. After 4 hours, ampoules were filled with 2.5 ml medium with or without HECNU and transfered into the microcalorimeter. Duplicate cultures for cell counts and analysis of other parameters were kept in an incubator. Cultures with an initial cell density of $0.6\text{-}0.8 \times 10^5$ cells/ml were used for measurement of HP and metabolic events.

2.2. Microcalorimetry

Calorimetric assays were performed with the LKB Bio Activity Monitor (BAM) 2277 (Pharmacia-LKB, Sweden), a thermopile heat conduction calorimeter [10], fitted with four ampoule measuring cylinders. Heat production was measured under static conditions at 37° C. HP generated per culture (μW) was recorded as a power-time curve (p-t curve). Values for heat production at zero were obtained by extrapolation of the p-t curve to this point. The stability of the base line was ± 0.46 μW for > 72 hours.

2.3. Determinations of pH,oxygen, glucose and lactate contents in the medium and intracellular ATP

The variables were determined from reference cultures four times during the experiments with 24 hours intervals.

Oxygen content of the medium was measured polarographically with an Orion electrode (Colora Meßtechnik, Lorch, FRG). The medium was sucked off from the closed glass ampoules with a gas-tight syringe and injected into a measuring chamber which containes the oxygen electrode. After measurement of O_2-content ampoules were opened and the medium from 2-3 reference ampoules per trial was collected in a 10 ml centrifugation tube. pH was determined immediately with a WTW pH meter (WTW, Weilheim, FRG). Medium was deproteinized with ice could 1.6 M perchloric acid. The supernatant was neutralized with a saturated solution of K_3PO_4. Glucose and lactate were determined by enzymatic methods [11-12]. Determination of ATP was performed as described earlier [8] by the luciferin-luciferase-system [13] in a LKB-Wallac luminometer 1251 (Pharmacia-LKB, Sweden).

HECNU was generously provided by Chemiewerk Homburg, Zweigniederlassung Degussa AG, FRG. It was used in concentrations of 0.1, 1.0, 10.0 and 100.0 μg/ml medium, respectively.

Statistical calculations were performed by the Wilcoxon test and by multiple regression analysis.

3. RESULTS

The cytostatic effect of HECNU measured as the number of population doublings (pd) during the 72 hour culture period approached significantly lower values with concentrations of 10 μg/ml HECNU and more as compared to control culture. This event was associated with an increase in the number of dead cells (Fig. 1).

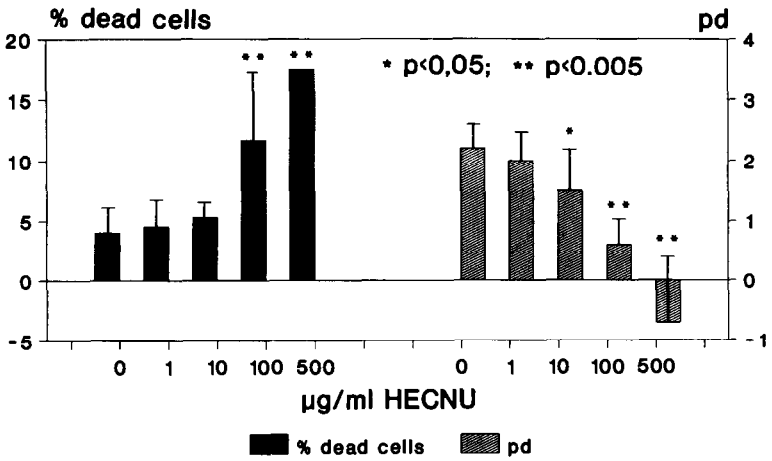

Figure 1. Effect of different concentrations of HECNU on cell survival (expressed as % dead cells) and population doubling (pd) after a culture period of 72 hours.

TABLE 1:
Effect of HECNU on HP (pW/living cell). Mean±SD; number of experiments in ( ).

[HECNU] (μg/ml)	control	1.0	10.0	100.0	500.0
0^h	47.9 ±12.6(6)	42.2 ±25.0(2)	53.4 ±15.7(3)	65.0 ±6.5(4)	50.1 ±6.6(3)
24^h	43.2 ±5.7(5)	40.4 ±16.5(2)	46.9 ±5.8(3)	47.0 ±8.2(4)	36.3 ±7.8(3)
48^h	32.4⊛ ±6.7(6)	27.8 ±2.4(2)	43.8 ±13.8(3)	56.3** ±11.6(4)	45.1* ±4.5(2)
72^h	20.2⊛⊛ ±7.6(6)	16.3 ±0.5(2)	28.5 ±19.1(3)	52.0* ±19.3(4)	41.9* ±5.8(3)

Significance with initial value in the control experiments: ⊛ $p<0.05$; ⊛⊛ $p<=.005$
Significance with corresponding controls:* $p<0.05$; ** $p<0.005$

HP steadily decreased in the control cultures throughout the experiment, the decline beeing significant after 48 hours (32%) and 72 hours (58%) (Table 1). Treatment with a low amount of HECNU (1 μg/ml) revealed a similar course whereas higher concentrations of HECNU resulted in maintained or even increased values compared with the corresponding control values (Table 1). In all cultures, a negative correlation was found between cellular HP and cell density (Fig. 2).

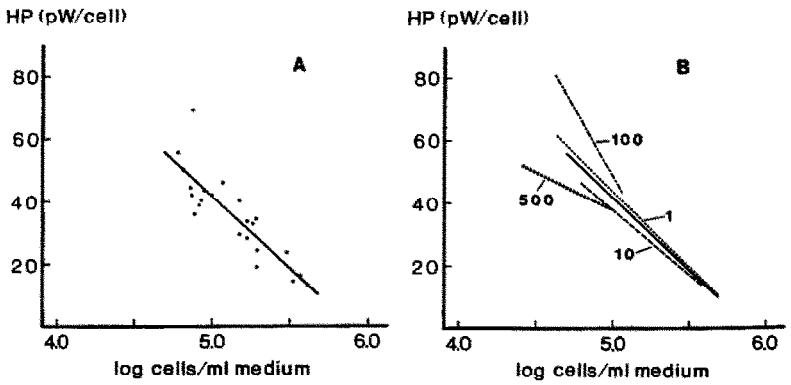

Figure 2. Correlations between HP/living cell and cell number in control (——), r= -0.87 (A) and in cultures treated with HECNU (B): (·····) 1 μg/ml, r=-0.77; (- - -) 10 μg/ml, r=-0.82; (-·-·-) 100 μg/ml, r=-0.71; (·····) 500 μg/ml, r=-0.53.

The effect of HECNU on pH and oxygen consumption at completion of the experiment expressed as the difference of pH value and the percent decrease of oxygen in the medium as compared with the initial values is given in Fig.3. Apparent changes were only detectable with the highest concentration (500 μg/ml) of the drug.

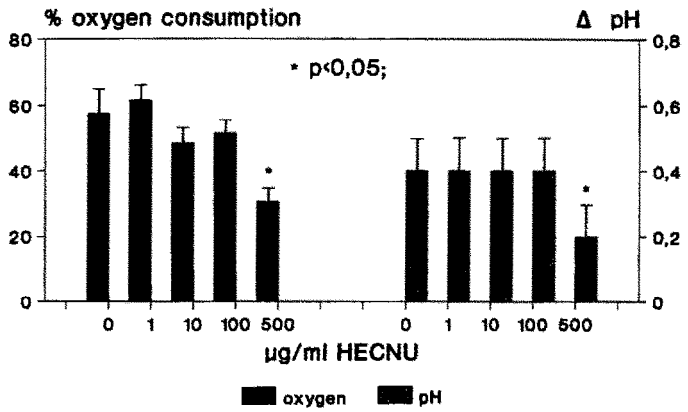

Figure 3. The effect of increasing concentrations of HECNU on oxygen consumption and pH at completion of the study as compared to the initial values.

As a measure for glycolytic conversion the concentration changes of glucose and lactate in the medium were calculated in three 24 hour periods by considering the initial values and those obtained at 24, 48 and 72 hours, respectively. The decrease of glucose and the increase of lactate concentrations were than divided with the arithmetic means of the corresponding cell numbers aquired from the same intervals. The sum of the mean values of these ratios may represent concentration changes over the entire 72 hours study period and are illustrated in Fig.4. Treatment with HECNU revealed no significant differences of these derived measures of lactate and glucose metabolism as compared with the controls.

The production of lactate per mole of glucose during the 72 hours period (Table 2) was similar in treated and untreated cultures.

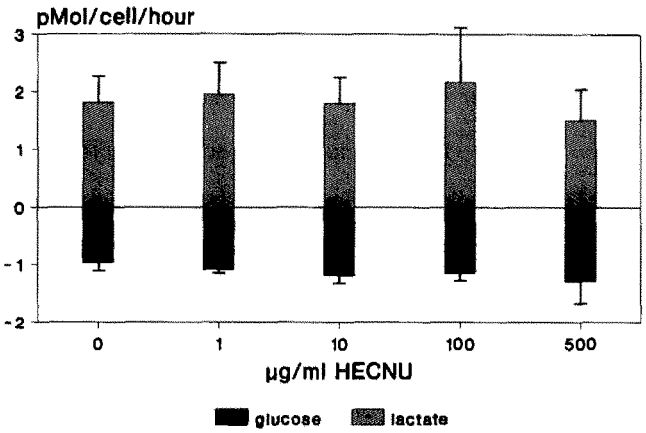

Figure 4: The measure of glycolytic conversion in treated and untreated cultures (explanation c.f. the text).

TABLE 2:
Production of moles lactate per mol of glucose

[HECNU] (µg/ml)	0	1	10	100	500
lactate prod. / glucose cons.	1.8±0.2 (6)	1.7±0.2 (2)	1.7±0.4 (3)	1.8±0.7 (4)	1.5±0.3 (3)

Irrespectively of treatment with HECNU cellular ATP contents did not differ significantly from the initial or control-values during the culture period.(Fig. 5).

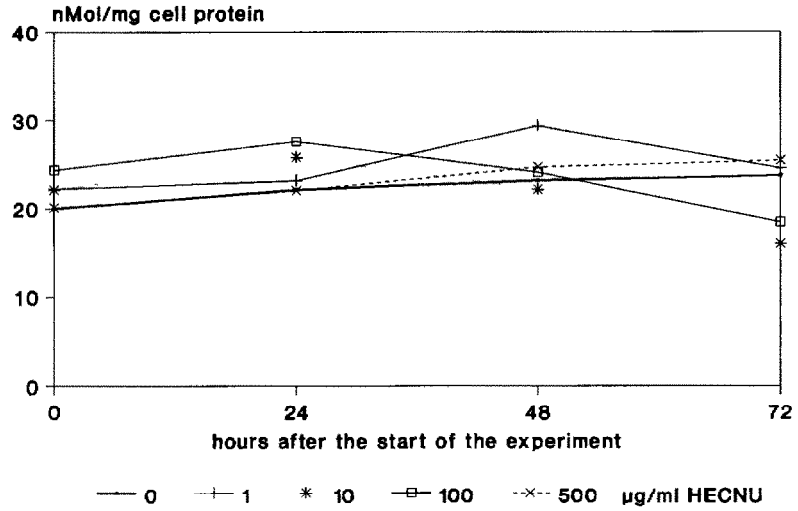

Figure 5. Intracellular ATP-content in control and in HECNU-treated cultures.

4. DISCUSSION

A major problem inherent with the use of chemotherapeutics in tumor research is their low specificity to the tumor cells. Thus harmful secondary effects on other cells and tissues may considerably complicate the proper interpretation of metabolic effects observed. HECNU is a haloethylnitrosourea with alkylating effect on nucleic acids and therefore retarding DNA synthesis by DNA-DNA interstrand cross-linkig [14-15]. According to our knowledge HECNU has not been used in clinical praxis [14] contrary to other nitrosoureas like BCNU [15]. Cumulative toxic side effect especially on the lung tissue [16] and possible carcinogenigenicity [17] were however reported following application of nitrosoureas while HECNU was suggested less toxic as observed in animal experiments [15,18-19].

To avoid metabolic differences due to different cell densities, measurements of HP and intermediary metabolism were only carried out with cultures in which an initial cell density of 0.6-0.8 x 10^5 cells/ml was obtained.

The phenomenon describing an initial decrease of HP with increasing cell densities has been previously described as the so called "crowding effect" [20]. Such a "crowding effect" apparently also occurs in our untreated cells of line IMR 90 during the growth period. Our investigations additionally demonstrate that this "crowding effect" apparently persists over the entire 72 hours culture.

It is generally assumed that "crowding-effect" is due to a lack in oxygen availability [21-22]. In the present study however due to the preferential anaerobic glycolysis oxygen consumption was low indicating that the availability of oxygen might not be the major factor in causing "crowding-effect". A possible proposal for the observed "crowding-effect" in our cells may be a decrease in nutrient availability and/or an increasing acidosis in the medium.

Interestingly in cultures treated with high concentrations of HECNU no "crowding-effect" was observed despite of a similar decrease in pH as seen in untreated cultures. One may speculate whether the absence of the "crowding-effect" is due to a higher nutrient availability since in these cultures the cell density is lower than in controls because of a cytostatic effect of HECNU.

A certain cytotoxic effect of HECNU is to assume when considering the increase of dead cells with enhanced content of the drug. An examination of the HP and HECNU concentrations however reveals no dose-response effect indicating that HECNU has little if any influence on the thermogenesis. Similarely glycolytic activity or cellular ATP-content were not affected by HECNU administration. Thus the results may suggest that the cytotoxicity of HECNU is not due to an influence on energy metabolism but tentatively affecting nucleic acid metabolism via the alkylating effect of the drug [16].

The method presented here apparently enables the assessment of the direct effect of pharmacon on cellular heat production. It obviously also allows certain coutious conclusions about the tolerance of the cells against the drug. We believe that the method as proposed in the present study may become a valuable alternative method to animal experimentation.

5. ACKNOWLEDGEMENTS

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