RATE OF SELECTION OF HIGHLY METASTATIC AND RESISTANCE-DEPRESSING TUMOR CELL VARIANTS *IN VIVO*

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The problem of what distinguishes tumor cells forming metastases from the other cells in the tumor population still remains unsolved. There is evidence in the literature [3, 5] to suggest that metastatic variants of tumor cells preexist in the population as genetic variants, which undergo selection *in vivo*. According to some data [7], metastatic variants of tumor cells may perhaps be transient phenotypic variants. Although cells of metastases are known to differ frequently from cells of the main tumor in many characteristics, the properties of tumor cells which determine their metastatic activity (MA) remain virtually unstudied. One approach to the study of this problem is to determine the factors of selection of metastatic variants of tumor cells *in vivo*. According to Gorelik et al. [4], variants of tumor cells artificially selected *in vitro* and *in vivo* on the basis of resistance to the cytotoxic activity of natural killer cells possess higher MA. The present writers have shown [1, 2] that during the formation of spontaneous lung metastases in Syrian hamsters with a subcutaneous tumor (strain STHE) variants of tumor cells which (unlike the original cells) have the ability to depress natural resistance (resistance depressing activity, RDA) of the animal to tumors and which have high MA, takes place. Under these circumstances correlation is found between the two above-mentioned characteristics of tumor cells. In that investigation cells of the STHE line (Syrian hamster embryonic cells spontaneously transformed *in vitro*) were used, for they possessed the following important advantages: 1) STHE cells cultured *in vitro* did not undergo selection *in vivo*; 2) they have no RDA and low MA; 3) they do not contain specific transplantation tumor antigens and, consequently, they cannot be selected *in vivo* with respect to this feature; 4) when animals are inoculated with these cells, they grow to form a tumor (sarcoma), which infrequently and irregularly metastisizes in the lungs of the tumor-bearing animals. With the discovery of difference in RDA and MA of metastatic variants of STHE cells from the parental STHE strain, the question arose whether these variant cells pre-exist in the original STHE population or whether they arise as new genetic variants during growth of the tumor or of its metastases *in vivo*.

The aim of the present investigation was to study the following problems: 1) Does selection of pre-existing metastatic variants of tumor cells take place *in vivo*; 2) Is proliferation of injected tumor cells *in vivo* an essential conditions for such selection? A new approach was used to the study of these problems: intravenous injection of STHE cells into animals, removal of these cells from lung tissue before the formation of visible lung metastases (usually formed on the 24th-27th day), growth of the cells *in vitro*, and the study of their RDA and MA.

EXPERIMENTAL METHODS

Tumor cells of the parental STHE strain were grown as monolayer cultures, removed from the glass with versene solution, and resuspended in Eagle's nutrient medium with 2% bovine serum, and injected as a single-cell suspension into the retro-orbital venous sinus of adult Syrian hamsters. Each animal received an injection of $5 \cdot 10^6$ to $6 \cdot 10^6$ tumor cells in a volume of 1 ml. The animals were anesthetized 1, 2, 5-6, 9-10, 14-16, and 20-21 days after injection of the tumor cells, their lung tissue was removed and minced under sterile conditions and suspended in versene solution, centrifuged, and the cell residue was resuspended in 1 ml of nutrient medium. This cell suspension was then layered on a Percoll

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TABLE 1. Determination of Rate and Efficiency of *in Vivo* Selection of Highly Metastatic (MA) and Resistance-Depressing (RDA) Variants of STHE Cells (before appearance of visible lung metastases)

Parameter	Time of transplantation of cells in vitro, days after injection in vivo					
	1	2	5—6	9—10	14—16	20—21
MA	1/10 (5 times)	2/12 (5 times)	4/6 (5-7 times)	2/6 (5 times)	4/6 (5-100 times)	4/6 (10-20 times)
RDA	0/10	0/12	0/6	1/3	2/6	2/6
Beginning of multiplication in vitro (in days) after isolated from lungs	10,0	9,5	7,5	5,0	4,1	4,1

Note. MA) Metastatic activity 5-100 times above control level (parental STHE cells). Numerator gives number of variants with the particular property, denominator number of variants studied.

gradient (to isolate STHE tumor cells, whose relative density is 18-24% of that of the Percoll solution, from the mixture of cells). The tumor cell fraction thus obtained was washed in nutrient medium to remove Percoll and transferred to a Leighton's tube for culture. Tumor cells from one animal were added to each individually numbered tube. A sample count showed that the number of tumor cells transferred to one tube varied from less than 10^4 up to 10^5. The beginning of growth of the tumor cells was noted for each sample of material. Cells removed from the lungs and giving growth *in vitro* multiplied and their RDA and MA were investigated simultaneously (as a rule at the 5th or 6th passage *in vitro*). The method of investigation of RDA and MA was described in detail previously [1, 2].

RESULTS

At different times after injection of STHE tumor cells into the animals, tumor cells isolated from the lung tissue of 155 Syrian hamsters were transplanted *in vitro*. Cultures from 43 of these animals were successfully propagated, and their RDA and MA were studied at the 5th or 6th passage. Multiplication of tumor cells *in vitro* after extraction from lung tissue begain in every case (but 1) not immediately, but 3-20 days after explanation, and only in 1 case was growth of tumor cells observed 24 h after transfer *in vitro*. The time of beginning of growth of the tumor cells *in vitro* was found to depend on the length of their stay in lung tissue *in vivo*: the shorter this stay, the longer their period in the steady state without multiplication *in vitro*. Results of experiments to study multiplication and RDA and MA of all 43 tumor cell variants isolated from the animals' lung tissue are summarized in Table 1.

The data in Table 1 show that selection of highly metastatic variants of tumor cells begins virtually immediately after injection of the tumor cells *in vivo*, and that by the 5th-6th day most variants of STHE cells still surviving *in vivo* possess higher MA. Resistance-depressing variants of the tumor cells could be found starting with the 9th-10th day after intravenous injection of STHE tumor cells. Just as in previous investigations [1, 2], in most cases direct correlation was found between the MA and RDA levels of individual variants studied.

Thus tumor cells, injected into the blood stream of animals and later removed from their lung tissue (where most of them settled during the first 10 min) at different times after injection, but before the appearance of macroscopically visible metastases, may differ from the parental cells with respect to two properties: ability to depress the natural resistance of the animal to tumors and ability to metastasize. Judging by the MA level, selection of highly metastatic tumor cell variants *in vivo* evidently begins as early as in the first 1-2 days after injection of the tumor cells, and by the 5th-6th day these variants can be discovered in the overwhelming majority of animals. Since the test which we used to determine RDA is much less sensitive than the highly sensitive test for determining MA, correlation between these two characteristics of the tumor cells becomes evident only in the later stages of selection (9th-10th day), when the RDA level of the test variants of tumor cells becomes higher. Since MA and RDA levels of tumor cell variants isolated in the late stages after injection of the cells were higher than at the early times of investigation and, as was shown previously, they become higher still if metastatic growths are investigated [1, 2], it will be evident that selection of tumor cells with respect to these features begins immediately after injection of the tumor cells *in vivo* and continues throughout the period before and after metastasis formation. It will also be evident that

all tumor cells injected into the blood stream are exposed to a powerful cytostatic influence, which evidently causes rapid death of the overwhelming majority of them [6]. The few surviving tumor cells, after multiplication and subsequent study at the population level, differ from the population of parental STHE cells quite consistently (i.e., for at least 5-10 passages *in vitro*) in their MA and RDA values. It must therefore be assumed that the surviving tumor cells, which possess these properties, evidently pre-exist in the heterogeneous population of parental cells as rare genetic variants, possessing selective advantages *in vivo*. As a result of *in vivo* selection the tumor cell population becomes significantly richer in these variants.

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HYDRAZINE SULFATE AS A CELL MEMBRANE STABILIZER

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The discovery that hydrazine sulfate (HS) possesses experimental antitumor activity, its distinctive beneficial effect in clinical oncology [1, 4, 9], and the absence of any marked side effects such as are characteristic of most antitumor agents [1, 2], have attracted attention to the study of the mechanism of its action.

The aim of this investigation was to study the action of HS on the stability of erythrocyte, mitochondrial, and lysosomal membranes and also of artificial phospholipid membranes.

EXPERIMENTAL METHODS

Experiments were carried out on mature rats obtained from the Rappolovo Nursery, Academy of Medical Sciences of the USSR.

The action of HS on the cell membranes was judged by determining hemoglobin release from erythrocytes, and in the study of the organelles, by activation of marker enzymes under the influence of factors modifying membrane function. Subcellular structures were isolated by differential centrifugation of liver homogenate [5, 6]. Mitochondrial ATPase activity or lysosomal acid phosphatase (AP) activity was determined and preparations of these enzymes were isolated as described in [6, 7].

To obtain erythrocytes the rats were decapitated and the blood collected and mixed in the proportion of 1 ml with 3 ml of 0.15 MNaCl solution, containing 1 U heparin, cooled to 4°C. The suspension of erythrocytes from each animal was divided into four equal portions. Two samples were incubated at 25°C for 60 min, after which HS was added to one sample (experimental) in a concentration of 10^{-4} M (with a uniform distribution of HS in the body fluids, this concentration corresponds to a sessional therapeutic dose of 60 mg/kg). After incubation at 25°C the samples were cooled for 60 min at 4°C. Two other samples were frozen at −15°C for 60 min; HS was added to one of the samples in the same concentration.

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