# HETEROGENEITY OF MOUSE SARCOMA CELLS FOR TUMORIGENICITY DUE TO DIFFERENCES IN THE DEGREE OF CONTACT INHIBITION OF CELL DIVISION

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One of the most important problems in the study of the multistage process of malignant transformation of cells is the isolation of the simple features from which it is formed. The solution to this problem, in the first place, would explain the details of the transformation process, and second, it would enable the tumorigenicity of cells to be predicted by weighing the contribution of each simple feature to their neoplastic potential. One cause of the slow development of this field of research is the difficulty of isolating cell lines with contrasting manifestations of single transformation features, due to the mosaic character of the tumor cells with respect to these features, because they are independently acquired [4]. The most effective method of isolation of these lines is to obtain clones having separate contrasting features; the difference between them so far as the remaining mosaic of features is concerned is, as a rule, minimal.

The aim of the present investigation was to isolate cell clones of mouse sarcoma differing in tumorigenicity, due to different degrees of contact inhibition of cell division.

## EXPERIMENTAL METHODS

Male and female CBA mice aged 2-3 months, bred in the Laboratory of Experimental Animals, Institute of Cytology and Genetics, Siberian Branch, Academy of Sciences of the USSR, were used. Cell sublines FCBA2V8 and FCBA2V10, maintained by passage in vitro, were obtained from embryonic fibroblasts of CBA mice, transformed spontaneously in vitro [2]. The first of them has gone through 8 passes in vivo, the second through 10. These sublines and 14 clones obtained from them were subcultured in vitro on Eagle's medium with 10% bovine serum. The clones were obtained in 60-well "Terasaki" plates by the limiting dilutions method. The cultures were free from mycoplasmas, as shown by incorporation of tritiated uridine and uracil [13]. Contact inhibition of cell division was assessed by the number of cells growing on a unit of standard plastic substrate [6]. The cells were counted on the 5th day after monolayer formation, and the medium was changed every day. The sensitivity of the cells to the lytic action of normal killer (NK) lymphocytes and macrophages was estimated by the degree of their lysis by these effectors in vitro. The degree of the immune reaction of the recipients to the corresponding cells was estimated by the cytotoxic test in vitro, using cells from regional lymph nodes and spleens of the corresponding recipients as effectors. The cytotoxic test was carried out in 96-well plates (Flow Laboratories, England) in accordance with the principle described by Mantovani [9], i.e., the degree of release from the target cells of tritium incorporated by cells on the eve of the test. All experiments to assess these characteristics of the cells were carried out repeatedly and the results were pooled and subjected to statistical analysis by Student's test.

## EXPERIMENTAL RESULTS

The starting point of the investigation was data on the high lability of sarcoma FCBA2V8 cells in the expression of tumorigenicity, as shown by its rapid loss during subculture of the cells in vitro (cells FCBA2V8† Table 1) and its recovery after a minimal number of subcultures in vivo (cells FCBA2V10, see Table 1). This last result indicated heterogeneity of this cell population, and this was confirmed by testing 14 clones obtained from the FCBA2V10 culture

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TABLE 1. Transplantability and Rate of Growth of Cell Clones, Isolated from FCBA2V10 Culture in Syngeneic Animals

| Cells | Weight of tumor (in g) 30 days after transplantation of cells in doses of | | | | Index of tumorigenicity |
|---|---|---|---|---|---|
| | $10^6$ | $10^5$ | $10^4$ | $10^3$ | |
| FCBA2V8 | 6,8±1,2 (100/75) | — | — | — | 12,2 |
| FCBA2V8 † | 0 (0/0) | — | — | — | 0,001 |
| FCBA2V10 | 11,6±0,9 (100/100) | — | — | — | 23,2 |
| Clones: | | | | | |
| 1 | 12,3±0,8 (100/100) | 11,3±1,4 (100/66,7) | 8,6±0,8 (100/7,7) | 3,3±0,7 (100/16,6) | 24,6 |
| 3 | 11,2±0,7 (100/85,7) | 8,8±1,2 (100/16,7) | 6,0±0,7 (100/0) | 2,0±0,6 (100/0) | 21,3 |
| 5 | 9,4±1,8 (100/50) | 7,7±0,8 (100/50) | 5,8±0,5 (100/16,7) | 5,6±0,1 (100/0) | 14,1 |
| 11 | 7,1±0,3 (100/54,5) | 5,4±0,4 (100/0) | 2,2±0,6 (100/0) | 0,6±0,1 (100/0) | 10,6 |
| 6 | 6,1±0,8 (100/33,3) | 5,4±0,9 (100/16,7) | 2,2±0,5 (91,7/0) | 1,0±0,3 (100/0) | 8,1<br>0 |
| 2 | 4,0±0,8 (100/0) | — | — | — | 4,0 |

Legend. Here and in Table 2: *) subcultured in vitro under 30 days, †) the same, more than 90 days. In parentheses: numerator — percentage of animals with tumors; denominator — percentage of animals dying before time of sacrifice; index of tumorigenicity = A·K1·K2, where A is the mean weight of the tumors (in g) on the 30th day (in the absence of tumors, this weight was conventionally taken as the minimal, i.e., 0.01 g), K1 the proportion of animals dying before the 30th day (varies from 2 to 1), K2 the fraction of animals in which tumors were formed by the 30th day (varies from 1 to 0.1).

TABLE 2. Transplantability and Rate of Growth of Cell Clones, Isolated from FCBA-2V10 Culture in Syngeneic Animals

| Clones: | Weight of tumor (in g) after transplantation of cells in a dose of $10^6$ | | Index of tumorigenicity |
|---|---|---|---|
| | after 30 days | after 60 days | |
| 9 | 0,01 (53,3/0) | 0,6±0,2 (100/0) | 0,005 |
| 10 | 0,01 (30,7/0) | 3,4±0,7 (100/0) | 0,003 |
| 4 | 0 (%) | 2,2±0,6 (68,8/18,8) | 0,001 |
| 7 | 0 % | 3,4±0,9 (90/10) | 0,001 |

for tumorigenicity. As regards their ability to take successfully, these clones formed a continuous series from highly malignant, killing all the animals in the course of 30 days, to weakly malignant, not forming tumors during this period (Tables 1 and 2).

To find the feature limiting growth of the weakly malignant clones, specific and nonspecific immune reactions of the recipients to cells of the above clones were assessed first, and the ability of the cells to exhibit contact inhibition of division, loss of which is an essential condition for autonomous growth [7, 9], was determined next. Careful weighing of the specific immune reaction of the syngeneic recipients against cells of two pairs of clones with contrasting tumorigenicity showed the absence of such inhibition to any of them (Fig. 1). The absence of an immune reaction of the recipients against these clones was confirmed by our experiments in vivo to determine transplantability of these clones in animals with depressed immunity. Data on sensitivity of the cells of the 10 clones mentioned previously to the lytic action of NK lymphocytes and macrophages are given in Fig. 2. The variability of these two features in the clones tested was minimal, and this was reflected in the low coefficients of correlation of these features with tumorigenicity (Fig. 2). Thus these data ruled out any participation of the immune system as a factor inhibiting tumor formation by weakly malignant clones.

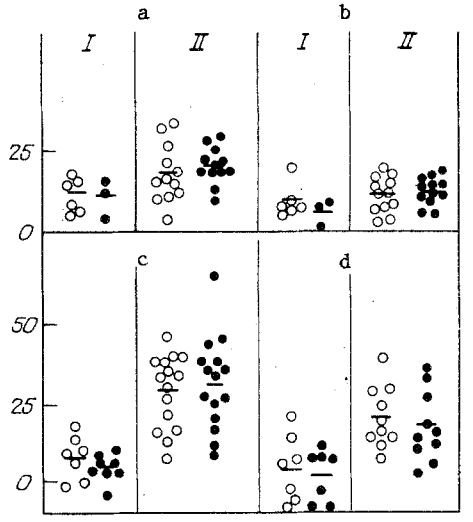


Fig. 1

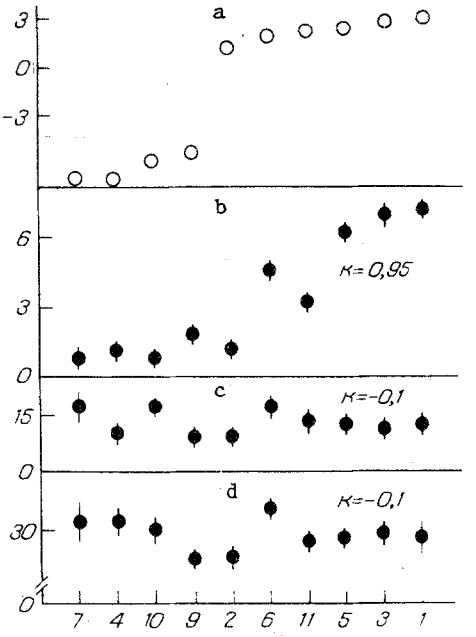


Fig. 2

Fig. 1. Cytotoxic activity of cells from regional lymph nodes (I) and spleens (II) of CBA mice on 9th day after transplantation with cells of clones 1 (a), 5 (b), 7 (c), and 4 (d). Ordinate, degree of lysis of corresponding target cells (in %). Open circles — control animals; closed circles — animals into each limb of which $10^6$ of the corresponding cells were transplanted. Ratio of target to effector 1:300, duration of reaction 48 h. In all cases $p > 0.1$.

Fig. 2. Tumorigenicity (a), degree of contact inhibition of division (b), and sensitivity to lytic action of macrophages (c) and of NK lymphocytes (d) of clones FCBA2V10 culture. Abscissa, no. of clones; ordinate: a) index of tumorigenicity, b) number of cells/cm$^2$ ($\times 10^{-5}$), c, d) degree of lysis of target cells (in %). Macrophages isolated from peritoneal cavity of animals by method in [1]; ratio of target to effector 1:30, duration of reaction 48 h. Syngeneic lymph node cells activated with strong transplantation antigens, according to the principle described in [3], were used as NK lymphocytes; ratio of target to effector 1:300; duration of reaction 24 h.

A completely different picture was observed when the clones were compared for their ability to give stratified growth on a plastic surface. This feature in the clones studied showed a high degree of correlation with tumor-forming ability: $K = -0.95$ (Fig. 2). The least malignant of these clones gave only monolayer cultures, whereas the more malignant, under the same conditions, formed satisfied tissue and gave a "yield" on the same area one order of magnitude greater than the weakly malignant clones. Cells of the weakly malignant clones, while indistinguishable in their morphology from the highly malignant variants in low-density culture, altered their morphology considerably in a monolayer and, unlike the latter, could survive in this state for a long time without a change of medium. Disturbance of control of cell proliferation is known to be a necessary but insufficient sign of malignant transformation [12]. Very often this sign does not correlate with the degree of transformation [5, 10, 14]. As already stated, the cause of this is the mosaic pattern of the tumor cells with respect to many features which together constitute the tumor phenotype [4]. However, our data show that given the correct choice of material, it is possible to obtain clones of cells whose degree of malignancy in the "pure" form depends on the degree of expression of the feature mentioned above. By contrast with other cell models, not cloned [5] or even cloned [11], and characterized by instability of this feature, in the clones which we obtained this feature was preserved in the course of many passages in vitro.

The clones which we obtained are thus a convenient model not only for the weighing of this feature in the transformed phenotype of the cells, but also for the comprehensive study of regulation of cellular proliferation during intercellular interactions.

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