Two Dynamic Morphotypes of Sarcoma Cells, Asymmetric Stellate and Triangle with Leading Lamella, are Related to Malignancy

(rat sarcoma / malignancy / pH / cell morphology / cell motility / dynamic morphotype)

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Abstract. A notion of the dynamic morphotype was developed as a conjunction between cell shape and migration. This enabled the investigation of the relationship between malignancy and patterns of dynamic morphology in neoplastic cells *in vitro*. Time-lapse cinemicroscopy was used to analyse the cell behaviour of three rat neoplastic cell lines (K2, T15, and A8), differing in metastatic potential, that were instrumental in revealing a coincidence between high migratory activity and appearance of the 3D structure of actin cables in high-malignant A8 cells (Pokorná et al., 1994). A set of criteria was established for visual classification of cell morphology. Matching the pattern of cell morphology with locomotory activity led to identification of four dynamic morphotypes. Cell speed was determined by tracking and the dynamic morphotypes assigned by the operator. All the three cell populations were studied for incidence of the dynamic morphotypes in culture media differing in pH: 6.6 simulating acid extracellular condition in tumours, physiological 7.4, and alkaline 8.2. The results showed that acid pH stimulated motile activity in the intermediate-malignant T15 and most malignant A8 cells. The T15 and A8 cells also manifested a prolonged continuation of fast locomotion in the early G1 phase and displayed a prevalence of two fast moving dynamic morphotypes: asymmetric stellate and triangle with leading lamella.

A formal analysis of histopathological diagnosis of malignancy revealed the importance of attributes related to the apparent locomotory activity of neoplastic cells (Veselý et al., 1985). As a consequence we have developed a spontaneously metastasizing rat sarcoma (Veselý et al., 1987) intended for investigation of tumour cell behaviour *in vitro* as related to *in vivo* malignancy. Several cell populations, all derived from clonal line LW13K2 (further K2) isolated as spontaneous transformants from mixed embryo fibroblasts of inbred rat strain LEWIS (Veselý and Weiss, 1973), were studied. Low-malignant clonal line K2, when injected subcutaneously, in spite of yielding tumours (10 x 6 x 6 cm) in all rats inoculated, only occasionally manifested one or two lung metastases in up to 1 out of 10 rats with tumours (Veselý et al., 1987). The T15 clonal line gave rise to 2–3 metastases in 40% (Veselý et al., 1987) and the A8 cell line produced 10 or more metastases in 80% of rats with tumours (Pokorná et al., 1994). The K2, T15 and A8 cells were analysed for the 3D organization of actin cytoskeleton (Pokorná et al., 1994). It was found that K2 cells expressed stress fibers, while for high-malignant A8 cells, a three-dimensional network of fine oblique actin fibers was typical. The T15 cells of intermediate malignancy showed a diffuse subcortical actin layer. No actin bundles were observed. These results posed a question about a possible relationship between malignancy, cell morphology and locomotory activity. To this aim the time-lapse film recordings of K2, T15, and A8 cell migratory behaviour in media differing in pH, slightly acid pH 6.6 for simulating the extracellular pH in a tumour (Ashby and Cautab, 1966; Stubbs et al., 1992), physiological pH 7.4 and slightly alkaline pH 8.2, were evaluated using contemporary tools of computer analysis. We have presumed that the results of this analysis could contribute to the interpretation of the pattern of cell behaviour observed by phase contrast optics in the time-lapse mode particularly in primary cultures from human tumours and thus enable more precise evaluation of their heterogeneity.

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Material and Methods

Cell populations

Three neoplastic cell lines K2, T15 and A8 were used, all of them were from the family of spontaneous-

ly metastasizing rat cell lines of mesenchymal origin differing in metastatic potential (Veselý et. al., 1989).

K2 – a clonal cell population of rat embryonic neoplastic cells. It was derived *in vitro* from spontaneous neoplastic transformants that appeared in a population of mixed embryo cells (mainly fibroblasts) from inbred rat strain LEWIS (Veselý and Weiss, 1973). The incidence of spontaneous metastases in lungs of inbred LEWIS rats shed by subcutaneous sarcoma tumours formed after subcutaneous injection of K2 cell suspension was 11% (Pokorná et al., 1994).

T15 – a clonal sarcoma cell population developed from K2 cells by neoplastic progression *in vivo* and *in vitro* (Veselý et al., 1987). The incidence of spontaneous lung metastases shed by subcutaneous T15 tumours was 43% (Pokorná et al., 1994).

A8 – a sarcoma cell population also developed from K2 by neoplastic progression *in vivo* followed by *in vitro* selection. Subcutaneous A8 tumours produced spontaneous lung metastases at the highest rate 85% (Pokorná et al., 1994).

Culture methods and preparation of cells for cinemicroscopy

Cells were seeded in Dulbecco's modification of Eagle's MEM supplemented with 10% calf serum (basic medium) (ZVOS Hustopeče, a.s., Uherčice, Czech Republic) in 60 mm culture dishes. After 24 h incubation, medium was replaced by the test media set to pH 8.2, 7.4 or 6.6 and the dishes were incubated for 30 min in a CO_2 incubator before the start of time-lapse recording. For the setting of pH, 20 mM HEPES (pH 8.2 and 7.4) or 6 mM PIPES (pH 6.6) was added to the test medium (without phenol red) and pH finally adjusted by 0.3 M NaOH or 1 M HCl.

Cinemicroscopy technique & evaluation

A field with five to ten single cells was found and recorded by time-lapse cinemicroscopy at intervals of 4 min for 24 h. An Olympus IM2 microscope was equipped with a 16 mm BOLEX camera. KODAK negative b/w film was used. A phase-contrast objective 10x gave a field width 800 μm. The cells were kept in controlled environment at 37˚C and flushed with 5% CO_2 (Riddle, 1990). Cell movement was at first analysed from frames at a 40 min interval. A Motion Analysis Projector (Spectro LTD, Windsor, England) was used. Cell outlines and trajectories were manually copied onto transparent paper, measured and entered into the database. Later, all films were digitized and computer tracked at full-time resolution of a 4 min interval. Mathematica 2.0 (Wolfram Research inc., Champaign, IL) was then used for data analysis.

Results

Classification of static cell morphology (Table 1)

Five categories were defined as non-transitional forms on the basis of the simplified cell shape and estimated height of the cell body and type of peripheral activity.

Round morphology is typical for cells during mitosis, with a circular cell outline without protrusions and lamellae. The phase contrast image of the cell shows a strong "halo" as a result of increased difference of refractive indices between the cell and the surroundings due to increased cell height. All round cells were mitotic (generation time 19–25 h), in the cultures studied no apoptotic or otherwise dying cells were observed by time-lapse microscopy.

Polygon is used for large flat cells with a polygonal outline active in small-scale ruffling and without polarized leading lamella.

Spindle morphology has a high prolonged cell body and two short protrusions on the opposite sites showing minimum membrane activity at the tips. The polarized leading lamella is missing.

Star morphology has a higher cell body and more than two protrusions with active tips. The protrusions have variable shape and length.

Triangle is used for cells with a higher body, fan-shaped leading lamella actively ruffling and a differentiated retraction tail.

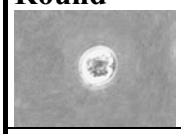
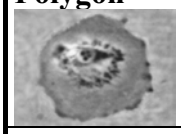
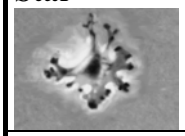
Influence of pH of the medium on cell migration (Table 2)

Slightly acid pH 6.6 stimulated, while slightly alkaline pH 8.2 inhibited cell migration. The average speed of moving cells increased with increasing malignancy and decreasing pH. The most malignant A8 cells showed the highest mean speed in slightly acid pH 6.6. The lowest speed was observed for the least malignant K2 cells in alkaline pH 8.2.

Traverse through cell cycle & motile activity (Fig. 1)

Early G1 cells showed the highest motile activity compared to the mean activity measured during the entire lifetime of the cells. Throughout the cell cycle the high activity decreased, and before further mitosis the cells became almost immobile. This was confirmed for all cell populations studied. However, in high-malignant cells the postmitotic active motile phase was proportionally about twice longer than in low-malignant cells.

Table 1. Classification and characterization of cell morphology

Morphology	Height	Leading lamella	Peripheral membrane activity	Cell processes
Round 	+++	A	-	absent
Polygon 	+	A	+	absent
Spindle 	+++	A	+/-	two short, thick protrusions on opposite sites
Star 	++	D	++	more than two protrusions with active tips
Triangle 	++	B	+++	retracting tail opposite to leading lamella

The height of a cell was estimated in phase contrast imaging using three categories on the basis of outline "halo": thin flat cells 1 to 2 μm high (+), intermediate height of 3 to 5 μm without bright "halo" (++), 5 to 10 μm high cells with notable "halo" (+++). The leading lamella can be absent (A), distributed (D) or broad (B). The peripheral membrane activity can be absent (-), rare (+/-), low (+), medium (++), or dramatic (+++).

Relationship between malignancy & cell morphology & pH (Table 2)

The polygonal morphology was characteristic for low-malignant and low-motile K2 cells in alkaline conditions. The spindle morphology was found in intermediate-malignant T15 cells, in low-malignant K2 in pH 7.4 or in high-malignant A8 in pH 8.2. The triangle or star morphology was observed for all cells in acid medium in a proportion increasing with malignancy.

Identification of the dynamic morphotypes (Fig. 2)

The difficulties with directly relating the cell morphology or migratory activity with malignancy led to the concept of the dynamic morphotype, which is understood as the combination of a repeating sequence of changes in cell morphology including transitional shapes that are associated with a distinct pattern of motility. The dynamic morphotype thus represents basic cell morphology, type of cell translocation and membrane activity indicating control of direction of locomotion. To the difference from static morphology, only four dynamic morphotypes could have been defined, as the round mitotic cells do not move. The roundish cells with short lamellae were considered too short-lived transitional forms in the cycle of a dynamic morphotype.

Triangle with leading lamella represents a cell of a triangular morphology with broad leading lamella moving directionally and briefly changing to a roundish morphology with a short leading lamella (Fig. 2a), with intermittent short intervals (4–10 min) of immobility in which the cell rests in an inactive spindle morphology.

Asymmetric stellate represents a cell with a star-shaped morphology. The position of the leading lamella is correlated with the direction of fast locomotion (Fig. 2b), while the remaining protrusions change continuously.

Stationary spindle represents a cell with a long spindle morphology slowly moving back and forth. Actual translocation is very limited. The two protrusions of the spindle morphology at the opposite ends show some activity.

Table 2. Incidence of cell morphology and speed of cell translocation in different pH of the medium

CELLS/pH	8.2	7.4	6.6
K2	POLYGON 88% 5 ±1.7 (35)	POLYGON 20% 3 ±2.2 (9); SPINDLE 19% 7 ±2.5 (17); STAR 5% 7 ±0.2 (5); TRIANGLE 37% 12 ±1.5 (53)	ND
T15	SPINDLE 57% 3 ±1.4 (63); TRIANGLE 34% 5 ±4.6 (58)	SPINDLE 15% 6 ±1.9 (21); STAR 27% 7 ±2.8 (34); TRIANGLE 49% 15 ±4.6 (59)	SPINDLE 19% 7 ±2.8 (6); STAR 17% 7 ±1.1 (6); TRIANGLE 65% 11 ±4.1 (16)
A8	SPINDLE 89% 6 ±3.2 (50); TRIANGLE 11% 8 ±0.9 (11)	SPINDLE 22% 9 ±8.7 (34); STAR 34% 12 ±3.5 (38); TRIANGLE 40% 28 +13.0 (63)	STAR 53% 22 ±13.7 (43); TRIANGLE 40% 31 ±18.2 (55)

The incidence is expressed as a fraction of cell observation time in %. Only incidences larger than 5% are included. Cell speed is presented as average ± standard deviation in μm/h followed by the number of measurements in brackets.

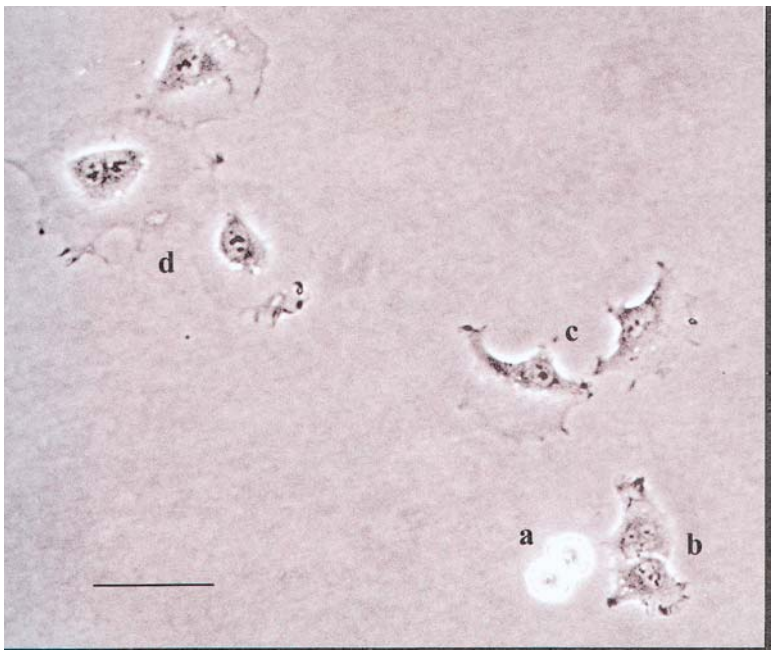


Fig. 1. Phase contrast micrograph of a colony of K2 cells illustrating the changes of cell morphology during the cell cycle. Cells have a round morphology during mitosis (a) and start to spread in early G1 phase (b). The cells in the second half of the cell cycle can have a triangular or polarized (c) morphology with leading lamella but prior to mitosis they change to the flat polygonal morphology (d). This is the most typical traverse through the cell cycle for the K2 cells. Scale bar is 100 μm.

Stationary polygon represents a cell of a flat polygonal morphology showing only a stationary although frequently active motion around the cell periphery without translocation.

Dynamic morphotypes and malignancy (Fig. 3)

Dynamic morphotypes combining morphological variability with locomotion were visually identified during computerized tracking and assigned to the cell trajectories (Fig. 3). This showed that their distribution corresponded to cell malignancy and pH of the medium. The most malignant A8 and T15 cells in pH 6.6 displayed a prevalence of the two highly motile dynamic morphotypes, asymmetric stellate and triangle with leading lamella, particularly in acid culture conditions.

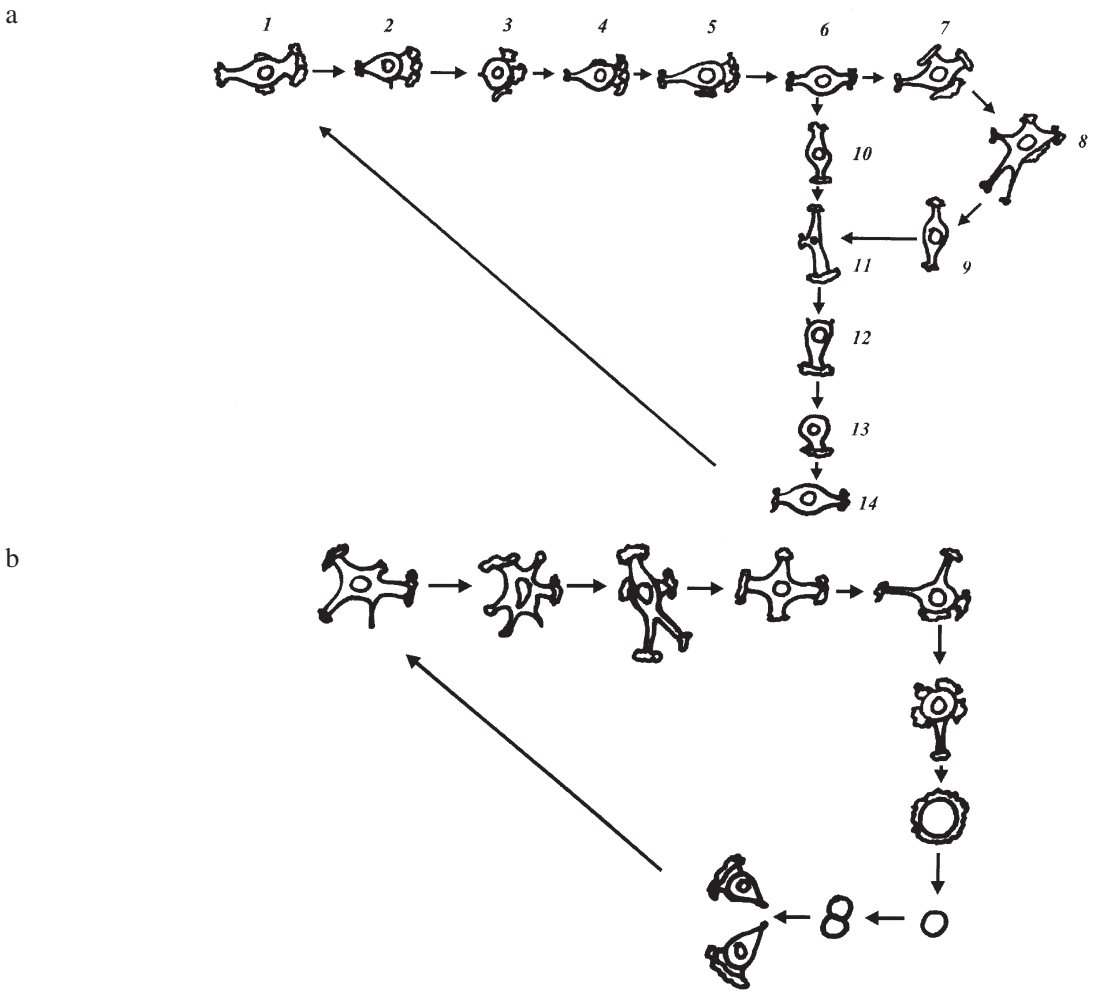


Fig. 2. The A8 cells with high-malignant potential displayed a prevalence of two fast moving dynamic morphotypes:
a) *Triangle with leading lamella*. The triangular cell in active locomotion moves forward by protrusion of the leading lamella (1). Then the tail is retracted (2) and a nearly round shape with a leading lamella is formed (3). This can be repeated several times (4, 5) and then the cell stays as a short spindle and does not move (6) for about 10 minutes. Next, the cell forms a new leading lamella on the other side of its body and continues to move in a new direction (10 – 13). The cell can also change its morphology and move as a star for some time (7, 8), and then again change to the triangle morphology (9, 11).
b) *Asymmetric stellate*. The cell moves fast in a directional way. There are three or more processes usually actively ruffling and the most active protrudes in the direction of the translocation. The shape of the cell outline changes more often than every 4 minutes while the cell has the star morphology and exhibits fast translocation.

Discussion

We analysed the morphology and migratory activity of cells from three neoplastic cell populations and the relation of these properties to malignancy. For the purpose of selection of highly metastasizing cell population needed for the study of the relationship between metastasis and *in vitro* cell migratory behaviour we had arrived to a two-step evaluation of malignancy. First, it was the incidence of tumour growth from standard inoculum. Second, it was the incidence and number of macroscopic metastases usually in the lung of diameter larger than 0.5 mm (Veselý et al., 1987). Our analysis complements former studies of actin cytoskeleton (Pokorná et al., 1994) and proteolytic activity (Chaloupka et al., 1998) by developing the notion of the dynamic morphotype and establishing its close relationship with malignancy. This approach can be important for a deeper insight into the diversity of primary cultures from spontaneous, particularly human, tumours and also for classification of the actual overall condition of the cell that is investigated for the fast intracellular motion (Veselý and Boyde, 2001).

Our efforts resulted in establishing the dynamic morphotype as a sequence of periodically changing cell shapes typical for a certain type of cell migration. Four dynamic morphotypes were identified. Finally, two of them, asymmetric stellate and triangle with leading lamella, were found to be associated with higher malig-

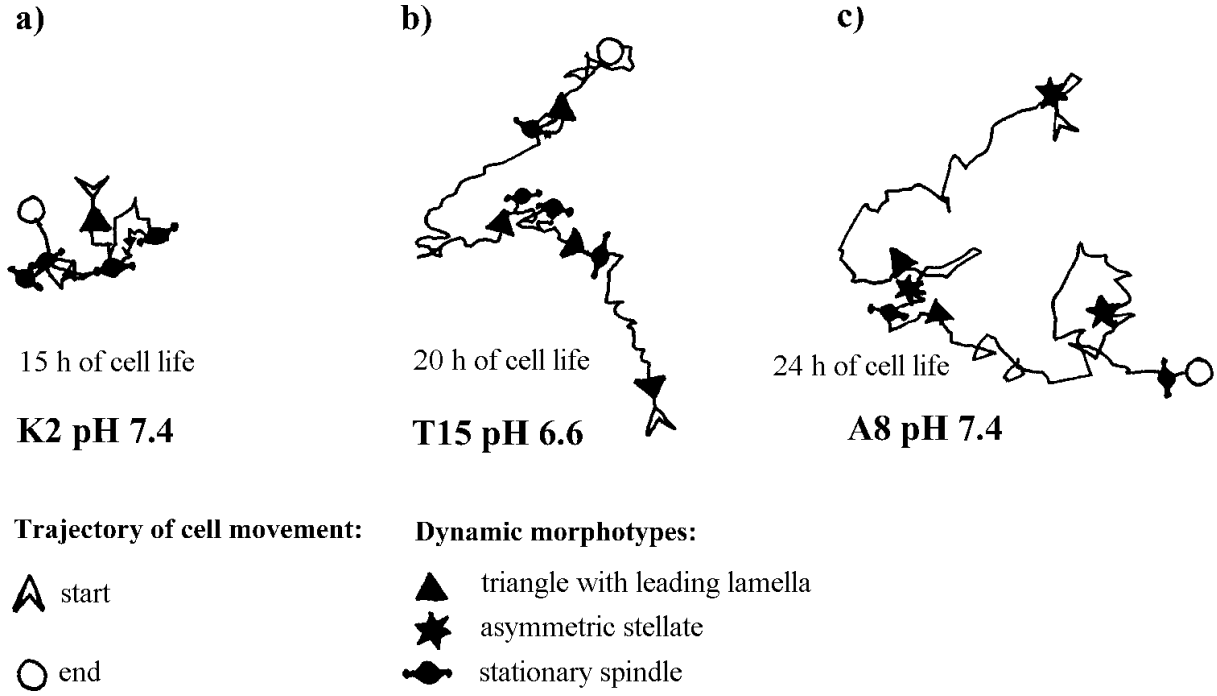


Fig. 3. Examples of trajectories illustrating the cell movement of three different cell types
The examples of trajectories illustrate the distance the particular cell type achieves and the changes of the direction of migration. The dynamic morphotypes are superimposed onto the trajectory to illustrate their incidence and frequency in the cell types studied.
a) A low-motile K2 cell of low malignancy in the pH 7.4 medium has a mainly ***stationary spindle*** dynamic morphotype, the cell was moving more or less in the same location (back and forth).
b) A T15 cell of intermediate malignancy with intermediate motile activity in the pH 6.6 medium has an increased incidence of a ***triangle with leading lamella*** dynamic morphotype.
c) An A8 cell of high malignancy with high motile activity in the pH 7.4 medium has a frequent ***asymmetric stellate*** dynamic morphotype. (In the pH 6.6 medium the motile activity of A8 cells is even higher – not demonstrated)

nancy. A similar mechanism of movement in the triangle with leading lamella was described by Sheetz et al. (1999) or Friedl and Brocker (2000).

Our analysis of *in vitro* behaviour of three rat sarcoma cell lines differing in metastatic activity showed that the shape of cells and type of cell movement in mutual coordination are typical for a particular cell line and are closely connected to its metastatic potential, confirming data from the literature (Geiger et al., 1984; Ben Ze'ev, 1987; Pokorná et al., 1994; Voura et al., 1998). The higher profile of the cell body and more active periphery of a cell evidently facilitate cell movement (Raz and Geiger, 1982). The morphology of cells also changes during the cell cycle. Daughter cells just after mitosis (early G1 phase) are higher, with increased motile activity, and usually have a star or triangle morphotype. In the course of growing old the cell motile activity decreases, the height of cells lowers and the shape changes to the polygon. Before next mitosis the cell is low, with decreased activity. In any cell population it is possible to find cells of several morphotypes which correspond to the time-points of traverse through the cell cycle. For this reason it is obligatory that control be exerted over as many variables as feasible.

We analysed the influence of acid and alkaline pH of culture conditions on migration and cell shapes. Changes in the morphology of cells cultured *in vitro* under different culture conditions represent a general phenomenon. The cells thus react to the changes in pH, temperature, medium composition or to the presence of toxic factors. Therefore, it is inevitable to control these parameters carefully in order to obtain meaningful results. The slightly acid pH of the medium was used to simulate the situation in the tumour. The pH can change the phenotype not only of malignant cells (Veselý et al., 1989), but also for example of fibroblasts during the healing of experimental wounds (Lengheden and Jansson, 1995). Similarly to the study of Martínez-Zaguilan et al. (1996) we showed that acid pH stimulates the cell locomotory activity. Our finding could be caused by the mechanism described by Martínez-Zaguilan et al. (1998), who reported that highly metastatic human melanoma C8161 cells have lower H+ buffering capacity than poorly metastatic A375P cells. In each cell line we studied there was a range of

morphotypes. The percentage of cells of a definite morphotype in a cell line varied. For example, the low-malignant K2 cell line contained a majority of polygons and a small number of stars or triangles, and among high round A8 cells it was also possible to find some polygons. The size of morphological fractions varied with malignancy and pH of the culture medium. The alkaline pH increased the incidence of polygonal cells and acid pH stimulated the incidence of stars and triangles.

These results, besides providing a new instrument for evaluation of the pattern of cell behaviour *in vitro,* also connote that in future such analysis should be complemented with simultaneous assessment of pHi/pHo (intracellular/outside pH) dynamics.

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