A Comparison of the Migration Patterns of Normal and Malignant Cells in Two Assay Systems

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The migration patterns of normal mouse embryo fibroblast (MEF) cells and mouse fibrosarcoma (FS) cells were compared in two assay systems. The two assay systems used were the modified Boyden chamber (micropore membrane) assay and the agarose drop explant assay. In both assays the major population of MEF cells exhibited a greater rate of migration than the major population of FS cells. However, a small subpopulation of FS cells which had a much greater rate of migration than the major population of either MEF or FS cells was detected in the agarose drop assay. A number of drugs which are known to inhibit the migration of leukocytes were tested against the MEF and FS cells. Concentrations were found that inhibited the major population of both groups by greater than 90%. However, at concentrations which inhibited the migration of the major population of FS cells by greater than 90%, a small group of fast-moving cells was still detected. Although the fast-moving cells were relatively resistant to treatment with the various drugs, this group was sensitive to a factor in serum. When normal human serum was used in place of fetal calf serum, the migration of the major population of FS cells was inhibited very little but movement of the fast-moving population was completely eliminated. We speculate that the small subgroup of fast-moving cells may be responsible for the invasive nature of the FS cells. (Am J Pathol 90:159–172, 1978)

RECENT STUDIES IN THIS LABORATORY have indicated that cells from several different tumors are capable of chemotactic responses to a cleavage product of the fifth component of complement.[1-3] The Walker carcinosarcoma cell, the murine mastocytoma cell, and the Novikoff hepatoma cell were used in this work. In extending these studies to other cell types, including a methylcholanthrene-induced fibrosarcoma of mice and normal mouse embryo fibroblasts, we observed significant differences between the migratory behavior of the normal and neoplastic cells in two different *in vitro* assay systems. This report describes the two assay systems used and the migratory behavior of the two cell types.

Materials and Methods

Tumor Cells

The fibrosarcoma cells (FS) used in this study were obtained from a tumor in C57 bl 6 mice; the tumor was induced with 3-methylcholanthrene. The tumor cells were grown in

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culture and maintained on a 50% mixture (v/v) of medium 199 with Hanks' salts and medium 199 with Earle's salts (M199) supplemented with 10% fetal calf serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. Cultures were grown at 37 C in a humidified atmosphere containing 5% CO_2. The cells were subcultured on a 3-day schedule at a split ratio of 1:4. The low split ratio and frequent subculturing schedule maintained the cells in a rapidly growing condition. By the tenth *in vitro* passage, the cells appeared morphologically homogeneous. When injected back into C57 bl/6 mice, a dose of 1×10^4 cells induced progressively growing tumors in nearly every animal.

Normal Cells

Swiss mouse embryo fibroblasts (MEF) were obtained from Microbiological Associates (Bethesda, Md). They were grown in M199 supplemented with 10% fetal calf serum and antibiotics. The cells were grown at 37 C in a humidified atmosphere containing 5% CO_2. To maintain the cells in as normal a condition as possible they were used in experiments only up through the first subculture.

Assays of Random Migration in Modified Boyden Chambers

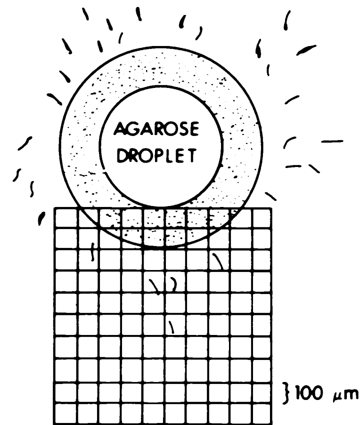
Modified Boyden chambers were used to assess random migration based on the method described by Romualdez and Ward for chemotaxis.[1] The chambers consist of two compartments separated by a filter. Cells to be tested were placed in the upper compartment at a concentration of 5×10^5 cells per chamber in M199 with serum. M199 with serum was also placed in the lower compartment. After 4 hours and 20 hours of incubation at 37 C in 5% CO_2, the filters were removed from the chambers, fixed in absolute propanol, stained with hematoxylin, dehydrated in propanol, and cleared in xylene. Cell migration was quantitatively assessed by light microscopy. The number of cells that migrated into the filters in five high-power fields (hpf) was counted. Selectron filters (Schleicher and Schuell, Keene, NH) of 8 μm porosity were used. Tests were performed in triplicate.

Assays of Random Migration Using the Agarose Drop Explant Method

The method used was a modification of methods described by Carpenter[4] and by Harrington and Stastny.[5] Cells to be tested were removed from culture dishes by tryspinization, washed, and centrifuged into a pellet. To 0.1 ml of the cell pellet was added 0.3 ml of M199 containing 10% serum and 0.2% (w/v) agarose (Seaplaque Agarose, Marine Colloids, Rockland, Me). Prior to use the agarose–cell suspension was kept in a water bath at 37 C; this prevented solidification of the agarose. One- to 2-μl droplets of the cell suspension were delivered with a sterile micropipette (Drummond microdispenser, Drummond Scientific Co., Broomall, Pa) into the wells of a microtiter culture dish (Linboro Scientific Co. Inc., New Haven, Conn). The microtiter dish was then placed in a refrigerator for 10 minutes to allow the agarose to solidify. After cooling, the agarose droplets were covered with approximately 0.3 ml of chilled M199 with 10% serum. The overlay medium was very gently added to the wells so as not to disturb the agarose drops. Tests were performed in quadruplicate. The microtiter plates were incubated at 37 C in 5% CO_2. Migration of the cells was examined daily for 1 to 5 days by phase contrast microscopy using an inverted tissue culture microscope. The microscope contained a calibrated grid in the eyepiece. The width of one grid space represented 100 μm actual distance at a magnification of 100×. The distance of the leading edge of migrating cells from the edge of the agarose droplet was determined on four sides of each droplet. Since tests were done in quadruplicate, this afforded us 16 measurements for each test (Text-figure 1).

Inhibition of Migration With Various Drugs

A number of drugs known to affect the migration of several cell types were tested for their effect on the motility of MEF and FS cells. The drugs were dissolved in M199,



TEXT-FIGURE 1—The migration of fibroblasts and fibrosarcoma cells from an agarose droplet. Cells migrate out of an agarose droplet onto the surface of a microtiter tissue culture dish. The width of the corona (the distance between the edge of the agarose droplet and the leading cell edge is defined as the width of the corona) of migrating cells is measured by placing the dish on the microscope so that a calibrated grid in the eyepiece is aligned with the edge of the agarose droplet. The number of grid spaces occupied by the corona is measured on 4 sides of each droplet. In addition, counts are made of the *number of isolated cells* which have migrated beyond the edge of the corona.

sterilized by filtration through 0.22-μm pore membranes (Millipore Corporation, Bedford, Mass), and serially diluted in M199. The diluted drugs were incorporated into the media in the agarose drop explant assay. The distance of migration of the cells in the presence of drugs was compared with migration distance in control media. In addition to measuring migration of the cells in the presence of the drugs, cells were also cultured in the presence of the drugs at the same concentrations. The cultures were examined daily, and concentrations that caused an alteration in the morphologic appearance of the cells were determined. In certain cases in which morphologic alterations in the cells were not observed even at high concentrations, growth rates of cells in the presence of drugs were compared with growth rates of controls.

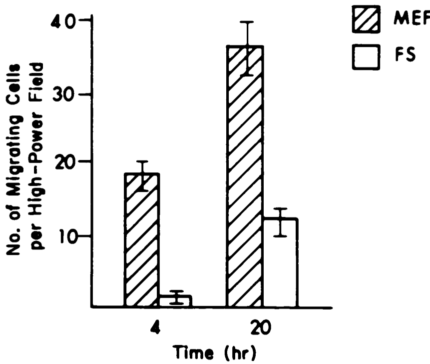
Results

Migration of FS and MEF Cells in Modified Boyden Chambers

FS and MEF cells were harvested from culture and their migration patterns were examined using Boyden chambers as described in *Materials and Methods*. Both the top well (with the cells) and the bottom well were filled with M199 containing 10% fetal calf serum. Membranes were examined after 4 hours and after 20 hours of incubation, and the numbers of cells migrating into the membranes were counted (Text-figure 2). At both 4 hours and 20 hours a significantly higher number of MEF cells than FS cells migrated into the membranes. The difference was particularly striking at 4 hours, when the average number of FS cells/hpf was less than 2 while the average number of MEF cells/hpf was 19.5.

Migration of FS and MEF Cells From Agarose Drop Explants

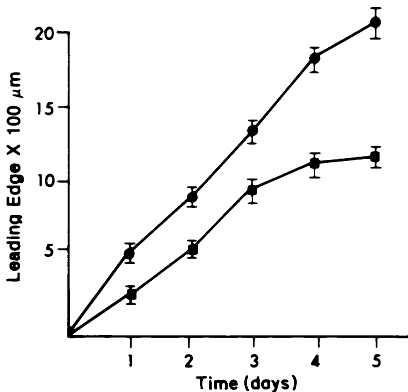
FS and MEF cells were harvested from culture and resuspended in agarose-containing medium as described in *Materials and Methods*. Agarose drop explants were prepared in microtiter dishes and the migration of cells from the explants was measured over a 5-day period (Text-figure 3).



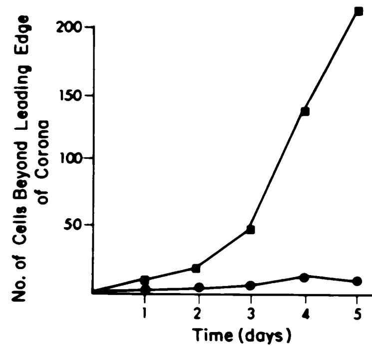
TEXT-FIGURE 2—Migration of fibrosarcoma (*FS*) and mouse embryo fibroblast (*MEF*) cells in Boyden chambers. The random migration of the two cell types in Boyden chambers was measured as described in *Materials and Methods*. The data are expressed as the number of cells that migrated into the membrane per high power field (hpf) ± standard error.

By the first day the leading edge of the MEF cell had migrated over 500 μm, while the leading edge of the FS cells had migrated less than 300 μm. Over the 5 days of observation, the leading edge of the MEF cells was always significantly further from the edge of the explant than the leading edge of the FS cells. By the fifth day the MEF cells had migrated over a distance of 2000 μm and the FS cells had migrated approximately 1200 μm. With both cell types the continued migration outward produced a uniform, expanding circular corona of cells around the central explant.

In addition to measuring the distance traversed by the leading edge, the cells that had migrated beyond the leading edge of the corona were counted each day (Text-figure 4). Although the corona of MEF cells expanded faster than the corona of FS cells, very few MEF cells migrated beyond the corona. Most of the MEF cells remained in contact with neighboring cells and those MEF cells that did migrate beyond the corona remained close to its edge. On the other hand, a large number of FS cells moved far beyond the corona. By Day 1 an average of 10 to 12 individual



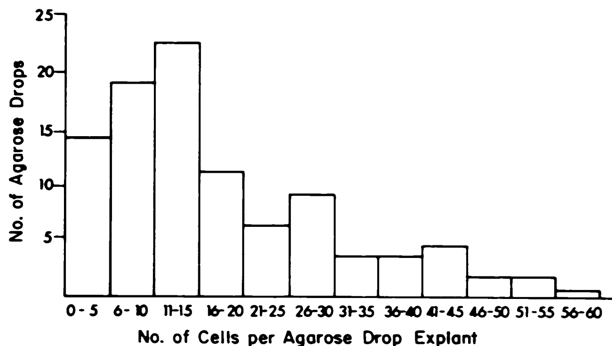
TEXT-FIGURE 3—Random migration of fibrosarcoma (FS) and mouse embryo fibroblast (MEF) cells out of agarose drop explants. Agarose drop explants were prepared in microtiter dishes as described in *Materials and Methods*. On each of the following days the migration of cells out from the drops was measured using an inverted microscope fitted with a grid in the eyepiece. Four readings were taken on each drop and four drops were used for each point (FS, ■—■; MEF, ●—●).



TEXT-FIGURE 4—Migration of fibrosarcoma (FS) and mouse embryo fibroblast (MEF) cells beyond the leading edge of agarose drop explants. Agarose drop explants were prepared in microtiter dishes as described in *Materials and Methods*. On each of the following days the migration of cells out from the agarose drops was measured (as described in Text-figure 3). In addition, the number of individual cells that had migrated beyond the leading edge was counted using an inverted microscope. (FS, ■—■; MEF, ●—●)

FS cells could be seen beyond the leading edge of the corona around each droplet. By Day 2 an average of 20 cells could be seen. A frequency distribution of cells beyond the corona on the second day after plating is shown in Text-figure 5. The data in this figure are based on observations of 113 individual agarose drops. The largest number of agarose drops had 11 to 15 cells beyond the leading edge. On the other hand, some had between 0 and 5 such cells and others had 51 to 60 cells beyond the leading edge of the corona. By Day 3 the average number had risen to 50 cells; by Day 5, an average of 200 cells was observed scattered over the surface of the dish beyond the corona. In contrast, 10 to 15 cells was the highest number of MEF cells observed beyond the leading edge on any day.

Since tumor cells are thought to have decreased adhesiveness as compared with normal cells,[6-8] it was possible that at least some of the FS cells seen far beyond the leading edge of the corona arrived there by detaching from the plastic surface and floating through the liquid overlay. To assess the possibility of this mechanism, an experiment was done in which agarose drop explants were covered with either the normal overlay (M199)



TEXT-FIGURE 5—Migration of the fast-moving population of fibrosarcoma cells beyond the leading edge of the corona. This histogram represents a frequency distribution of the number of fast-moving cells per agarose drop counted on the second day after plating.

Table 1—Replacement of the Normal Overlay With an Overlay Containing 0.2% Agarose

Overlay	Corona size (× 100 μm) ± SE	No. of cells migrating beyond corona
M199 + 10% fetal calf serum	6.4 ± 0.1	38
M199 + 10% fetal calf serum + 0.2% agarose	5.4 ± 0.1	46

or a M199 overlay to which was added 0.2% agarose. After 3 days of incubation the migration of cells in the two groups was compared (Table 1). The corona size was reduced slightly in the cultures treated with the semisolid agarose-containing overlay (550 vs 650 μm). However, the number of cells migrating beyond the corona was almost identical in both groups.

Replacement of Fetal Calf Serum With Human Serum

In certain agarose drop explant experiments the fetal calf serum in the overlay medium was replaced with human serum. Human peripheral blood was drawn immediately prior to use and allowed to clot. Fresh or heat-inactivated (56 C, one-half hour) human serum was used directly in the overlay medium. The effect of replacing fetal calf serum with human serum is shown in Table 2. Both normal and heat-inactivated human serum supported the migration of FS cells. The leading edge of the corona advanced at approximately the same rate in the presence of either normal or heat-inactivated human serum, similar to the results with fetal calf serum. A striking difference was noted, however, in the number of cells which migrated beyond the leading edge. In the group treated with heat-inactivated human serum, the number of cells observed beyond the leading edge was comparable to the number of cells beyond the leading edge in the fetal-calf-serum–treated group. In the presence of fresh human serum, the number of cells observed beyond the corona was reduced by approximately 85%, compared with the results obtained with either heat-inactivated human serum or with fetal calf serum. These results indicate that the movement of cells beyond the corona is not due simply to

Table 2—Replacement of Fetal Calf Serum in the Overlay Medium With Either Normal Human Serum or Heat-Inactivated Human Serum

Serum	Corona size (× 100 μm) ± SE	No. of cells migrating beyond corona
Fetal calf serum	4.2 ± 0.18	25
Normal human serum	3.1 ± 0.34	6
Heat-inactivated human serum	4.3 ± 0.25	23

an artifact in the preparation of the agarose drops but is a specific event under the control of serum factor(s).

Effects of Various Drugs on the Migration of FS and MEF Cells

Drugs that affect a variety of biochemical functions were tested for their ability to inhibit the migration of FS and MEF cells. The drugs included inhibitors of nucleic acid metabolism and protein synthesis (such as mitomycin C, puromycin, and cycloheximide),[9-11] agents that interfere with microtubules and microfilaments (such as colchicine, vinblastine, and cytochalasin B),[12-14] and cyclic nucleotides or agents that alter cyclic nucleotide levels.[15,16] The agarose droplet explant assay was used for these studies. For both FS and MEF cells the migration of cells within the corona was measured. In addition, the number of FS cells migrating beyond the corona was also assessed. With the exception of cGMP, all the agents used were able to inhibit the migration of both FS and MEF cells (Table 3). Inhibition of migration occurred in the range of concentrations that also induced visible alterations in the morphology of the cells. With the exception of mitomycin C, less than a 1-log difference separated the dose causing a visible alteration in the morphology of the cells and the dose causing a 50% reduction in migration of coronal cells. Complete suppression of migration did not normally occur until concentrations were reached which were much higher than this. Furthermore, even at concentrations which inhibited migration of coronal cells by more than 90% there was much less effect on the FS cells that migrated beyond the edge of the corona. Cytochalasin B suppressed the number of FS cells beyond the corona by nearly 90%, but the other drugs were much less effective (Table 4).

Discussion

In this study we have compared the migration patterns of normal MEF cells and mouse FS cells in two assay systems: the modified Boyden chamber assay in which cells migrate into micropore filters and the agarose drop explant assay in which cells migrate out of a concentrated suspension of cells in agarose onto the surface of tissue culture plates. In both assay systems the MEF cells migrated faster than the FS cells. This is in contrast to observations by Wood et al,[17] who showed that V2 carcinoma cells migrated much more rapidly than normal epithelial cells, fibroblasts, and monocytes *in vivo*. The disparity in methodology makes comparison of such observations difficult. These differences could also be due to differences in the type of tumor cells used. We have other tumor

Table 3—Effects of Various Drugs on the Migration of Fibrosarcoma (FS) and Mouse Embryo Fibroblast (MEF) Cells

Drug	Concentration causing toxicity (μg/ml)*		Concentration inhibiting migration† By < 10%		By 50%		By > 90%	
	FS	MEF	FS	MEF	FS	MEF	FS	MEF
Mitomycin C	5×10^{4}	1×10^{1}	1×10^{4}	1×10^{3}	2×10^{2}	2×10^{2}	1×10^{1}	1×10^{0}
Cycloheximide	8×10^{2}	8×10^{1}	3×10^{3}	4×10^{3}	2×10^{2}	5×10^{2}	5×10^{1}	1×10^{0}
Colchicine	1×10^{2}	1×10^{2}	1×10^{4}	1×10^{4}	4×10^{3}	4×10^{3}	1×10^{1}	1×10^{1}
Vinblastine	1×10^{4}	1×10^{4}	1×10^{5}	1×10^{5}	7×10^{4}	2×10^{5}	1×10^{1}	1×10^{0}
Cytochalasin B	7×10^{4}	—	1×10^{4}	—	5×10^{3}	—	5×10^{1}	—
cAMP	1×10^{2}	6×10^{1}	3×10^{1}	3×10^{1}	3×10^{2}	3×10^{2}	5×10^{2}	5×10^{2}
Theophylline	1×10^{3}	5×10^{2}	3×10^{2}	1×10^{2}	3×10^{1}	3×10^{2}	2×10^{3}	2×10^{3}
cGMP	NR	NR	5×10^{2}	5×10^{2}	NR	NR	NR	NR

* FS and MEF cells were grown in 25-sq-cm flasks in various concentrations of each drug. Cultures were observed microscopically daily for 3 days, and the lowest concentration of each drug that induced morphologic alteration in the cells during the observation period was considered to be the level causing toxicity. With theophylline and cAMP, alterations in the morphology of the cells were not observed at the doses used. The doses indicated in the first column represent the concentration of drug which inhibited the growth of cells by 20%.

† Inhibition of migration refers to inhibition of the corona of migrating cells.

NR = not reached in the range of concentrations between 1×10^{2} to 5×10^{2} μg/ml.

Table 4—Effects of Various Drugs on the Migration of the Fast-Moving Population of Fibrosarcoma Cells

Drug tested	Mean number of cells beyond the corona at drug concentration causing migration inhibition of coronal cells by: < 10%*	> 90%*
Mitomycin C	22†	10
Puromycin	20	10
Cycloheximide	20	12
Colchicine	46	12
Vinblastine	14	10
Cytochalasin B	17	2
cAMP	10	8
Theophylline	19	8

* The actual concentrations of each drug can be found in Table 3.
† Mean number of cells beyond the corona counted 2 days after plating.

lines which migrate in our agarose drop explant assay much faster than either the MEF or FS cells described here.[18]

In the 4-hour Boyden chamber experiment, 10 times as many MEF as FS cells migrated into the filters. By 20 hours there were 3 times as many MEF as FS cells in the filters. In the agarose drop explant assay, the leading edge of the MEF cell corona was always 40 to 60% farther from the edge of the agarose drop than the leading edge of the FS cells.

Although the MEF cells migrated at a faster rate than the FS cells when the major populations of the groups were compared, there was a minor population of FS cells which migrated far beyond the leading edge of the corona in the agarose drop assay. The FS cells observed beyond the leading edge of the corona constitute a very small percentage (less than 0.1% by Day 2) of the total number of cells originally present in the agarose drop explant. The number of cells seen beyond the corona is also somewhat variable. By Day 2 some of the agarose drops had as few as 1 to 2 cells, while in one explant .65 such cells were observed. The average number of cells in a survey of 113 individual agarose drop explants examined 2 days after plating was 20.6. A similar subpopulation of cells was not observed in the MEF cultures on any day. Whether the individual cells observed beyond the leading edge of the corona constitute a population with unique biologic properties is not known at present. We are studying the *in vivo* and *in vitro* properties of clones of the fast-moving cells and comparing these properties with those of the uncloned population.

Drugs that have been shown to affect the motility of leukocytes were tested for their effects on the FS and MEF cells in the agarose drop assay. It was possible to inhibit the migration of both FS and MEF cells with

certain of these agents. However, none of these agents inhibited migration at concentrations far below those concentrations that induced cytotoxic alterations or inhibited the growth rates of the cells. Furthermore, even at drug concentrations that induced cytotoxic changes in the FS cells and inhibited the migration of the corona of cells by 90%, a large number of individual cells was still observed to have migrated far from the edge of the agarose drop. The only drug tested which did effectively inhibit the migration of this population of cells was cytochalasin B, a drug which acts on the microfilament system.[14] The inability to inhibit the migration of these cells with high concentrations of the various chemical agents may be of practical importance as well as of theoretic interest since it may be that these fast-moving cells are responsible for the invasive nature of this tumor. On the other hand, the number of cells that migrated beyond the leading edge of the corona could be reduced by 80 to 90% when normal human serum was used in place of fetal calf serum. This implies that this characteristic can be modified under the appropriate conditions.

The migratory behavior in culture and the response of cells in culture to interaction with other cells have been studied. When normal fibroblasts come into contact with other normal fibroblasts, they respond by cessation of movement in the forward direction.[19,20] This inhibition of movement is referred to as "contact inhibition." Malignant cells have been described as being deficient in their response to contact with other cells.[21-25] Of particular importance in this respect is the observation that tumor cells show impared response to contact with normal cells. It has been reported that malignant cells migrating as whole groups and as detached individual cells were able to invade colonies of normal fibroblast cells.[25] It is possible that the FS cells we have observed migrating beyond the leading edge of the corona are the cells responsible for the invasive nature of these tumors.

The two assay systems were used in conjunction in this study because they measure parameters of cell migration which, although probably related, are not necessarily identical. Each assay has certain advantages and disadvantages. It would appear that maximum amounts of data can be obtained by combining the assays.

A major difference between the two assays is the length of the incubation period. In the Boyden chamber assay, a short incubation period (4 to 20 hours) is used; in the agarose drop explant assay, migration is assessed at daily intervals over a 1- to 5-day period. The short incubation of the Boyden chamber assay allows migration to be evaluated under conditions in which cell multiplication is minimal. In the longer incubation for the agarose drop assay, cell division does occur along with cell migration, and it is difficult to separate the two functions.[26-29] From a

purely practical standpoint, the short-term incubation of the Boyden chamber assays allows the assay to be completed in a single day while the agarose drop assay requires a much longer time.

Since the Boyden chamber assay uses such a short incubation period, the condition of the cells at the start of the assay is very important. We have found that FS cells migrate well when harvested and tested 1 to 3 days after being plated, whereas cells maintained in culture for longer periods respond much less well. Primary or secondary cultures of MEF cells should be used within 1 to 2 weeks after being plated. If they are maintained in culture for longer periods, they also respond less well. The decreased responsiveness of "old" cultures may be due to the fact that these cultures must be treated with trypsin for a longer period prior to harvesting. Furthermore, even after the longer period of trypsinization, it is difficult to obtain suspension of single cells, which is necessary for optimal movement into membranes. On the other hand, the condition of the cells at the start of the assay seems to be much less important in the agarose drop assay probably because with the longer incubation period the cells have much more time to adapt to the conditions of the assay.

Another major difference in the two assays is that in the Boyden chamber assay the membranes are fixed and stained before the migrating cells are counted. In the agarose drop method, migration is measured *in situ* without disturbing the progress of the incubation. The cells are still viable after the reading, and the rapidly migrating cells can be easily cloned by this method. To clone the fast-migrating cells the agarose drop is initially put onto a coverslip in a small Petri dish. After a sufficient time to allow the fast-moving cells to migrate off the edge of the coverslip, the coverslip is removed and the remaining cells are allowed to grow.

In summary, we have compared the migratory behavior of normal fibroblast and malignant fibrosarcoma cells in two assay systems. The migratory behavior of the major populations of both cell types was similar; the normal cells actually migrated somewhat faster than the tumor cells. We observed, however, a small subpopulation of tumor cells which migrated much faster than the major populations of either cell type. Drugs which are known to inhibit the migration of leukocytes were tested against both cells. The fast-moving subpopulation of FS cells was more resistant to most of these drugs than was the major population. The only drug that inhibited the migration of the fast-moving subpopulation to a degree comparable to that of the major population was cytochalasin B. On the other hand, the subpopulation of fast-moving cells was sensitive to a factor in serum. When normal human serum was used in place of fetal calf serum, the development of this subpopulation was not observed.

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