Characteristics of the Chemotactic Response of Neoplastic Cells to a Factor Derived From the Fifth Component of Complement

William Orr, MD, James Varani, PhD, and Peter A. Ward, MD

Chemotactic factors for malignant neoplastic cells can be generated from either the fifth component of complement or from leukotactic fractions obtained from zymosan-activated serum. Digestion of the fifth component of complement by trypsin initially produces leukotactic activity, but as digestion continues, leukotactic activity is lost and tumor cell chemotactic activity is generated. Separation of the leukotactic activity and tumor cell chemotactic activity can be accomplished by gel filtration or isoelectric focusing. Gel filtration indicates that the tumor cell chemotactic factor has a molecular weight of approximately 8000 daltons. Tumor cell chemotactic activity can be generated by trypsinizing the leukotactic fractions isolated by isoelectric focusing. The responses of cultured Walker tumor cells or of Walker ascites tumor cells are dose-dependent and truly chemotactic. Cells from a murine malignant lymphoma do not respond to the complement-derived chemotactic factor for tumor cells, indicating that not all malignant cells share this functional property. (Am J Pathol 93:405–422, 1978)

IT HAS BEEN SHOWN recently that factors derived from tumor tissue [1,2] and from the fifth component of complement [3] stimulate the locomotion of malignant neoplastic cells *in vitro*. There is evidence that this phenomenon may be an important mechanism by which metastasis occurs *in vivo*.[4] The studies conducted *in vitro* have utilized Boyden chambers, counting the number of cells that migrate into the pores of a micropore membrane and toward the stimulating factor. The tumor cell responses has been described as chemotactic, ie, the cells more toward an increasing concentration gradient of attractant. An alternative but not mutually exclusive possibility would be that the responses reflect enhancement of random migration, ie, chemokinesis. It has not been determined which of these mechanisms is mainly responsible for the movement of tumor cells in Boyden chambers.

A number of different tumor cell types have been observed to respond in chemotaxis chambers. Notably, these include cells from transmissible ascites tumors (including the Walker carcinosarcoma, Novikoff hepatoma, murine mastocytoma,[5] and ascitic forms of rat and murine hepatomas

From the Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut.

Supported by Grant AI 09651 from the National Institutes of Health.

Dr. Orr is a Fellow of the R. Samuel McLaughlin Foundation, Toronto, Canada.

Accepted for publication July 5, 1978.

Address reprint requests to William Orr, MD, Department of Pathology, University of Connecticut Health Center, Farmington, CT 06032.

0002-9440/78/0119-0405/$01.00

AH109A and MH134, respectively) and murine myeloid leukemia (C-1498).[1,2] These studies have inferred that enhanced migration or chemotactic movement of neoplastic cells may be a ubiquitous phenomenon, common to many cell types; no tumor cells have been reported to lack the ability to respond to chemotactic agents.

It has been demonstrated in this laboratory that factors capable of stimulating the locomotion of tumor cells can be generated from normal human or animal serum, zymosan-activated serum, or the purified fifth component of complement (C5). The chemotactic activity can be generated by treatment of whole serum with tissue extracts,[3] by incubation of activated serum with inhibitors of leukocyte chemotactic factors,[6] and by digestion of intact C5 with trypsin.[3]

The chemotactic factor for tumor cells cannot be generated in serum deficient in C5 or by treatment of the third component of complement (C3) with trypsin. Although the products of serum, activated serum, or C5, which contain activity for tumor cells, appear to lack chemotactic activity for leukocytes, the ability to generate activity for tumor cells from partially purified leukotactic peptides suggests that the tumor cell activity may be related to a cleavage product of the larger leukotactic peptide.[6] This is consistent with observations from sucrose gradient ultracentrifugation that the activity for tumor cells is located in fractions of lower molecular weight than the leukotactic factors.[3]

The data presented in this paper indicate that the locomotory responses of the Walker carcinosarcoma cells induced by complement products are dose-dependent and truly chemotactic. Evidence is provided that Walker tumor cells maintained in tissue culture or in the ascites form in animals demonstrate similar chemotactic responses. However, cells from a murine malignant lymphoma line do not respond to chemotactic stimuli. Physicochemical analysis of the complement-derived chemotactic activity for tumor cells indicates that it is different from the activity which is chemotactic for leukocytes, although the chemotactic factor for tumor cells may be derived from the leukotactic peptide. It is also shown for the first time that trypsinization of partially purified leukotactic fractions of zymosan-activated serum is a relatively simple method for the generation of chemotactic activity for tumor cells.

Materials and Methods

Ascites Tumor Cells

The Walker carcinosarcoma, maintained as an ascites tumor by serial passage, is the same tumor line used in previous studies.[3,5,6] Sprague–Dawley rats weighing 80 to 150 g received intraperitoneal injections of 2×10^7 cells. Four to five days later the animals were killed by ether anesthesia and the tumor ascites fluid was harvested by aspiration into a

syringe containing heparinized (10 IU heparin/ml) medium 199 without fetal calf serum. Following centrifugation at 750 rpm for 10 minutes, the supernatant was discarded and the cells were resuspended in 0.83% ammonium chloride to lyse erythrocytes. The cells were then washed three times in medium 199 by repeated centrifugation and resuspension in fresh medium. Finally, they were suspended in medium 199 supplemented with 10% fetal calf serum at a concentration of 4×10^6 cells/ml. This cell suspension was used for studies of chemotaxis.

Cultured Tumor Cells

The Walker carcinosarcoma, maintained by serial passage in tissue culture, was obtained commercially (Flow Laboratories, Rockville, Md.). The tumor cells were grown in a 50% mixture (v/v) of medium 199 with Hanks' salts and medium 199 with Earle's salts, 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 after trypsinization on a 4-day schedule at a split ratio of 1:5. The low split ratio and frequent subculturing schedule maintained the cells in a rapidly growing condition. The cells appeared morphologically homogeneous after the fifth passage following culture of cells obtained from Flow Laboratories. When injected by intramuscular or intraperitoneal injection into male Sprague–Dawley rats weighing 80 to 150 g, a dose of 1×10^6 cells induced progressively growing tumors in most animals. In addition to subculturing these cells on a 5-day schedule, selected flasks were not split on the fifth day but, instead, were permitted to grow to maximum density. This was accomplished by changing the culture medium on these cells at 3- to 4-day intervals without removing the cells by trypsinization. When this procedure was followed, the bottom surface of the tissue culture flasks was covered by a dense monolayer of cells by the fourth to fifth day after seeding the flask. By the seventh to eighth day, the density of the cells was such that many cells had begun to pile on top of the monolayer. For chemotaxis studies, the cells were removed from the flasks by agitation (rather than by trypsinization), centrifuged at 750 rpm for 10 minutes to remove them from the original tissue culture medium, and resuspended in medium composed of an equal volume of the original (conditioned) medium and fresh medium 199 with 10% fetal calf serum. The final cell concentration was 5×10^6 cells/ml, a concentration 10-fold less than that used for ascites tumor cells. If, following agitation to suspend the cells in the medium, the cells were placed in a new flask, more than 90% of the cells attached to the surface within 4 hours. Their subsequent growth in medium 199 with 10% fetal calf serum was similar to that of cells seeded into flasks after trypsinization.

The EL-4 murine malignant lymphoma line was kindly supplied by Dr. Nancy Ruddle at Yale University. This thymidine kinase-deficient tumor was grown in medium RPMI-1640 supplemented with 10% fetal calf serum, 1% glutamine, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. Flasks containing 20 ml of this medium were seeded with 2×10^6 EL-4 cells and incubated with the flasks closed at 37 C. The tumor cells grew in suspension and the cells were subcultured on a 4-day schedule. When injected intraperitoneally into C57/bl6 mice, 2×10^7 cells produced ascites tumors in virtually all animals. For studies of chemotaxis, cells were harvested from the flasks and removed from the media by centrifugation at 750 rpm for 10 minutes. They were then resuspended at a concentration of 1×10^6 cells/ml in medium composed of equal parts of the original (conditioned) medium and fresh RPMI-1640 supplemented with fetal calf serum, glutamine, and antibiotics.

Neutrophilic Leukocytes

Peritoneal exudates were induced in rabbits by injection of glycogen in saline as described by Cohn and Morse.[7] Leukocytes were harvested after 4 to 6 hours by paracentesis, centrifuged to form a pellet, and resuspended in Hanks' medium containing 0.7

mM Ca^{2+} and Mg^{2+}, 0.134 mM PO_4 anion, 0.74 mM SO_4 anion, 200 mg/dl glucose, 25 mM Tris buffer, and 0.05% bovine serum albumin. For chemotaxis assays the final cell concentration in this medium was 2.5×10^6 leukocytes/ml.

Preparation of C5

The purified fifth component of complement was prepared from normal human serum according to the method of Nilsson et al.[8] The purity of the material obtained was tested by immunodiffusion using goat antihuman C3, goat antihuman C5, and goat antinormal human serum. The purified C5 formed a single precipitation band with anti-C5 and antinormal human serum but not with anti-C3. In addition to analysis by immunodiffusion, electrophoresis in 4% polyacrylamide in the presence of 0.1% SDS buffer was performed. Using this method, results similar to those of Nilsson et al[8] were obtained; two paired bands, close to each other, migrated 20 to 30% of the length of the gels. The C5 was pooled, concentrated, dialyzed against phosphate-buffered saline, and stored at −70 C until use.

Leukotactic Fractions of Zymosan-Activated Serum

This material was prepared by a modification of the method of Vallota et al.[9] Normal human serum containing 1 M epsilon amino caproic acid was incubated with 1% (w/v) zymosan at 37 C for 1.5 hours. The zymosan was then removed by centrifugation (500*g*) for 15 minutes. Following adjustment of the pH to 5.5 by glacial acetic acid, the serum was applied to a Sephadex G100 column which had been equilibrated with 0.15 M sodium acetate buffer, pH 3.7. A broad zone of leukotactic activity was eluted and appeared in the latter portion of the protein profile. This area was pooled and stored in pH 3.7 acetate buffer at −70 C. The specificity and purity of the isolated chemotactic factor was determined by reaction with rabbit antibodies. The leukotactic activity of this material was blocked with rabbit antihuman C5 but not with antihuman C3. Before use, the preparation was thawed and neutralized with 0.05 M dibasic sodium phosphate.

Bacterial Chemotactic Factors

Escherichia coli culture fluids were prepared according to the method of Ward et al.[10] Cultures of *E coli*, selected for their ability to generate bacterial chemotactic factors (WHO reference strain 16), were grown for 24 hours in liquid culture medium. The bacteria were removed by centrifugation and filtration, and the culture fluid supernatant was used as a chemotactic factor.

Trypsinization Procedures

Purified C5 was treated with 1% trypsin (w/w) at 37 C in an agitating water bath. Samples were taken from the reaction mixture immediately following the addition of trypsin to C5 (zero time) and/or at the various times indicated in *Results*. Soybean trypsin inhibitor (2% w/w) was added to each sample to inhibit the action of trypsin at the end of the digestion.

Leukotactic fractions of zymosan-activated serum were treated with 1% trypsin (w/v) at 37 C in an agitating water bath. The trypsin dose was equivalent to 0.75% trypsin (w/w) based on the total protein concentration in the sample, determined by the method of Lowry et al.[11] Samples were taken from the reaction mixture at zero time and at the various times indicated in *Results*. Soybean typsin inhibitor (2% w/v) was used to inhibit the action of trypsin at the end of the digestion.

Assays of Cell Migration and Chemotaxis

Assays of cell migration and chemotaxis were done by a method slightly modified from that of Romualdez and Ward.[3] The micropore filters used for studies of tumor cell

migration were composed of nitrocellulose, with a porosity of 12 μ (Selectron filters, Schleicher and Schuell, Keene, N.H.). The filters used for studies of leukocyte chemotaxis were composed of cellulose acetate, with a porosity of 0.65 μ (Millipore Corp., Bedford, Mass.). After assembling the Boyden chamber, putative chemotactic factors in varying concentrations suitable for dose-response studies were placed into the lower compartment of the chamber in a total volume of 1 ml. In some experiments, these factors were also placed into the upper compartment with the cells to be tested to vary the concentration of factors on both sides of the micropore membrane. Control media consisted of appropriate tissue culture media alone. Tumor cells or leukocytes, suspended in the various media and at cell concentrations described above, were placed into the upper compartment of the chamber. The chambers were then incubated at 37 C in a humidified atmosphere. The latter contained 5% CO_2 for incubations of cultured tumor cells but was composed of room air for studies of ascites tumor cells and leukocytes. The duration of incubation was 1.5 hours for studies of leukocytes but was 4 to 4.5 hours for studies of tumor cells.

After incubation, filters were processed in the usual manner. Quantitation of the migratory response was performed by counting the numbers of cells per high-power field that had migrated from the surface of the micropore filter into the interstices of the filter. In each experiment three chambers were set up for each of the conditions to be tested. The migration of cells in three high-power fields was counted in each of the three filters. The mean and variance of the nine values obtained for migration into the three filters was calculated to determine the mean number of cells migrating per high-power field.

Checkerboard Analysis of Cell Migration

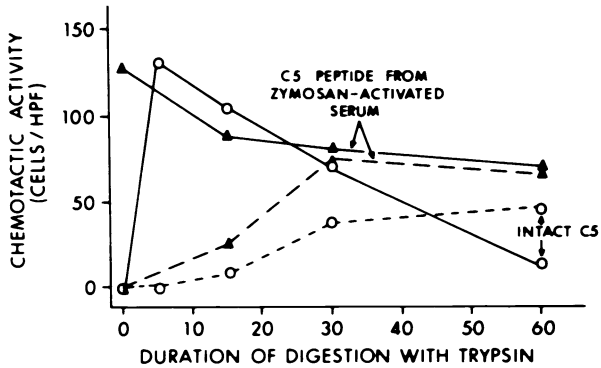
The method of Zigmond and Hirsch [12] was employed to determine if the locomotory responses of the neoplastic cells were truly chemotactic. Serial dilutions of trypsinized leukotactic fractions of zymosan-activated serum (30-minute digestion) were prepared over a concentration range which, on the basis of dose-response studies (see *Results*), was judged to include the dose of trypsinized activated serum required to elicit a 50% maximum response of the cells being tested. In addition to the usual preparation, various concentrations of trypsinized leukotactic fractions of zymosan-activated serum were added to the tissue culture medum used to suspend the cells so that, while the concentration of tumor cells and fetal calf serum remained constant, a number of cell suspensions were prepared, each having a different concentration of trypsinized leukotactic fractions of activated serum. The different concentrations of this stimulant in the cell suspensions corresponded to the concentrations prepared for injection into the lower well of the Boyden chambers.

Gel Filtration

Sephadex G75 (Pharmacia Fine Chemicals, Piscataway, N.J.) was prepared as directed by the manufacturer and poured into a column measuring 35 cm × 1.2 cm with a bed volume of 30 ml. The column was eluted with phosphate-buffered saline, pH 7.2, and calibrated with blue dextran (mw, 2×10^6), chymotrypsinogen A (mw, 2.5×10^4), myoglobin (mw, 1.7×10^4), cytochrome C (mw, 1.3×10^4), insulin (mw, 6×10^3), and phenol red (mw, 3.5×10^2). Aliquots of C5 varying in quantity from 520 to 1000 μg were trypsinized for 60 minutes and applied to the column in a volume of 1 ml. Aliquots of leukotactic fractions of zymosan-activated serum, measuring up to 1 ml in volume, were trypsinized for 30 minutes before application to the column. Fractions of 1 ml were collected and assayed for protein by the method of Lowry.[11] One hundred microliters of each fraction was diluted to a volume of 1 ml for chemotaxis assay.

Isoelectric Focusing

The method was modified from that of Radola.[13] Samples of C5 or leukotactic fractions of activated serum containing $\leq$ 1 mg of protein were trypsinized for appropriate periods.



TEXT-FIGURE 1—Generation of leukotactic activity and tumor cell chemotactic activity from C5 and from leukotactic fractions of activated serum. Purified C5 and leukotactic fractions from zymosan-activated serum were treated with 1% trypsin (w/w and w/v, respectively) at 37 C. At the times indicated, aliquots of the reaction mixture were removed and 2% soybean trypsin inhibitor was added to the aliquot. Each aliquot was then assayed for leukotactic activity and for chemotactic activity for tumor cells. *Solid lines*, leukotactic activity; *broken lines*, chemotactic activity for tumor cells; *circles*, activities derived from C5; *triangles*, activities derived from leukotactic fractions of activated serum.

One percent Ampholines (v/v) over a pH range of 2 to 11 (Brinkmann Instruments, Westbury, N.Y.) were added to each sample; these mixtures were then applied by centrifugation to glass-enclosed columns (12.5 cm × 0.5 cm) of Sephadex G75 superfine to which 1% Ampholines had been added during preparation of the gel. In some experiments the sample to be electrofocused was mixed with the gel slurry prior to pouring the gel into the column. Isoelectric focusing over a pH range of 2 to 11 was carried out by the application of a potential difference of 150 volts across the column for 4 hours. One-centimeter fractions of the gel were collected, 300 μl of water was added, and the pH of each fraction was determined. One millilter of 0.05 M phosphate buffer, pH 7.3, was added to each fraction. The supernatants of each fraction were assayed for absorbance in a spectrophotometer at 280 nm. One hundred microliters of each fraction was then diluted to 1 ml in Hanks' solution and tested for chemotactic activity.

Results

The Generation of Chemotactic Activity for Tumor Cells by Trypsinization of Purified C5 and by Trypsinization of Leukotactic Components of Zymosan-Activated Serum

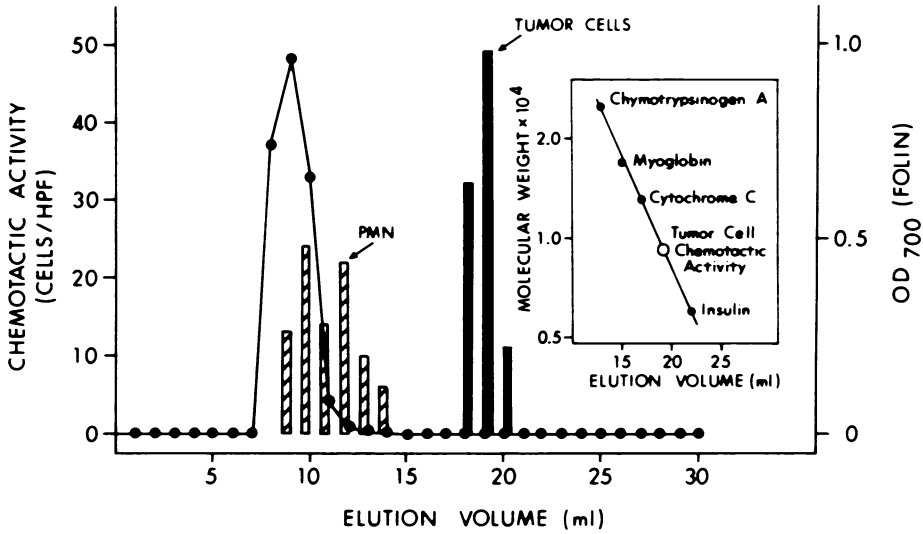
Purified C5 was trypsinized (5 to 60 minutes), and the digestion mixture was analyzed for chemotactic activity. Fifty micrograms of C5 was placed into each Boyden chamber. Samples taken at zero time (immediately following the addition of trypsin to the C5) failed to enhance the migration of either leukocytes or tumor cells (Text-figure 1). However, the aliquot taken after 5 minutes of digestion induced, as expected, migration of large numbers of leukocytes. Each sample of the digestion mixture taken subsequent to the 5-minute period induced fewer numbers of leukocytes to migrate than did the sample taken previously. Enhanced migration of tumor cells was first detected in the sample taken 15 minutes

after the initiation of trypsin treatment. This activity began to reach its maximum level in the 30-minute sample, increasing only slightly in the 60-minute sample.

Leukotactic fractions of zymosan-activated serum were trypsinized for similar periods. Aliquots of the digestion mixture were prepared for Boyden chamber assay so that the final concentration of substrate (100 μl/ml) induced a quantitatively similar cellular response to that caused by 50 μg of C5. Maximum stimulation of leukocyte movement was found in samples taken immediately following the addition of trypsin to the sample (time zero). Subsequent samples demonstrated progressively diminishing stimulation of leukocyte movement. However, the decrease in leukotactic activity over time was not as great as that seen in the digestion of intact C5. The ability of trypsin-treated leukotactic fractions from zymosan-activated serum to stimulate the migration of tumor cells was first detected in the aliquot taken after 15 minutes of digestion. This activity reached its maximum after 30 minutes of digestion. Thus, the activity which caused enhanced migration of tumor cells could be generated either from C5 or from the leukotactic factor in zymosan-activated serum. In both cases the appearance of tumor cell activity developed after the generation of leukotactic activity. The intensity of the stimulation of tumor cell movement continued to increase as the intensity of the stimulation of leukocyte migration decreased. The chemotactic activities for leukocytes and for tumor cells were both blocked by treatment of samples with antibodies to C5. The leukotactic activity generated by 5 minutes of trypsinization of 50 μg of C5 was reduced by 45% after treatment with 10 μl of heat-inactivated goat serum containing anti-C5. The same treatment blocked completely the tumor cell chemotactic activity generated after 60 minutes of trypsinization. Treatment of 100 μl of leukotactic fractions of activated serum with 40 μl of heat-inactivated goat serum containing anti-C5 blocked leukotactic activity by 83% and completely blocked the tumor cell chemotactic activity generated after 30 minutes of trypsinization.

Behavior in Gel Filtration of the Chemotactic Activity for Tumor Cells

Aliquots of trypsinized leukotactic fractions of zymosan-activated serum were chromatographed as described above. In three experiments, results similar to those depicted in Text-figure 2 were obtained. Residual leukotactic activity was located in fractions associated with or adjacent to the void volume of the column. The tumor cell chemotactic activity was regularly located in fractions with an elution volume of 18 to 21 ml, beyond the cytochrome C marker. Chromatography of trypsinized C5 on the same column produced virtually identical results; the two activities eluted from the column in the same positions as did activity in the

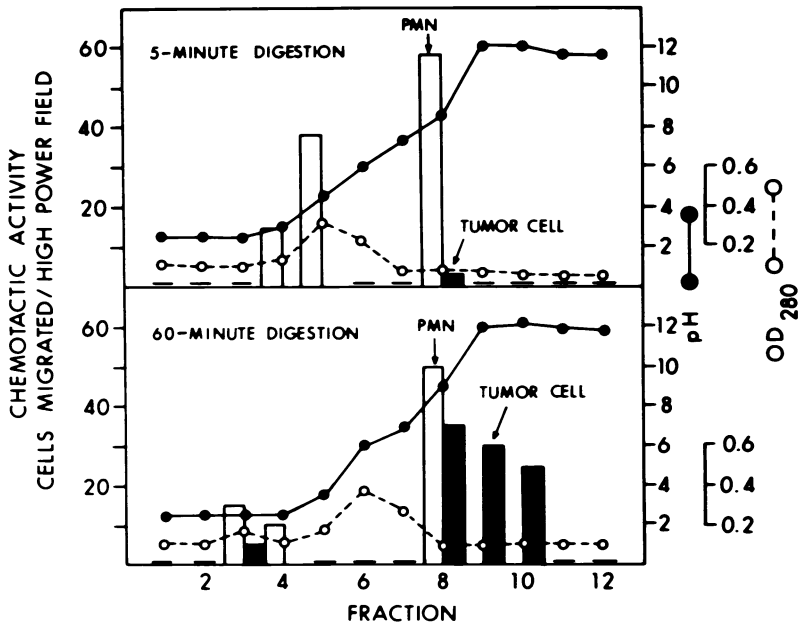


TEXT-FIGURE 2—Gel filtration on Sephadex G75 of zymosan-activated serum. Leukotactic fractions were trypsinized for 30 minutes under conditions described in the text. The column fractions were assayed for protein, for chemotactic activity for tumor cells, and for chemotactic activity for leukocytes (PMN). The column was calibrated with chymotrypsinogen A (mw 25,000), myoglobin (mw 17,000), cytochrome C (mw 13,000), and insulin (mw 6000).

trypsinized fractions from activated serum. When the elution position of the chemotactic activity for tumor cells (derived from either starting substrate) was compared with the elution position of several protein standards, the activity was found in fractions corresponding to a molecular weight of approximately $8 \pm 2 \times 10^3$ daltons.[14] The elution volume of the leukotactic activity corresponded to a molecular weight of $2 \pm 0.5 \times 10^4$ daltons.

Behavior in Isoelectric Focusing of the Chemotactic Activity for Tumor Cells

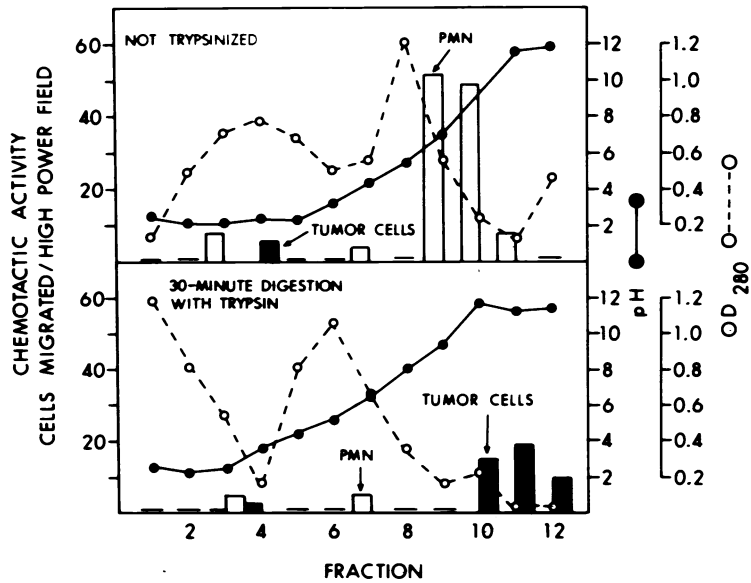
C5 was trypsinized for 5 minutes to generate leukotactic activity and was then isoelectrically focused as described in *Materials and Methods*. The results of one such experiment are shown in the upper frame of Text-figure 3. At a wavelength of 280 nm a peak of absorbance was detected between pH 4 and pH 5, corresponding to the isoelectric point of C5. A major peak of leukotactic activity was found between pH 7 and pH 9 in all experiments. In three of four experiments a second peak of leukotactic activity was located in the fractions between pH 4 and pH 5, corresponding to the peak of absorbance at 280 nm. A small amount of chemotactic activity for tumor cells was seen in all experiments in the same fractions as the major peak of leukotactic activity. In parallel experiments, identical quantities of C5 from the same lot were digested for 60 minutes to



TEXT-FIGURE 3—Isoelectric focusing of trypsinized C5. Purified C5, 860 μg, was digested for 5 minutes at 37 C with 1% trypsin (w/w). The reaction was stopped with 2% soybean trypsin inhibitor. A second sample was digested for 60 minutes under the same conditions. Each sample was then isoelectrically focused as described in the text. Fractions were assayed for pH absorbance at OD_{280}, chemotactic activity for tumor cells, and chemotactic activity for leukocytes.

generate chemotactic activity for tumor cells. In a typical experiment, depicted in the lower frame of Text-figure 3, a peak of absorbance at 280 nm was found between pH 4 and pH 5, corresponding to the peaks found in samples digested for 5 minutes. A peak of leukotactic activity was found in fractions between pH 7 and pH 9. A minor zone of leukotactic activity was also found in the most acidic portions of the preparation. Tumor cell chemotactic activity was found to overlap with the zone of chemotactic activity and to extend into the extreme alkaline pH region of the gradient. In two of four experiments employing purified C5, a small zone of tumor cell chemotactic activity was also found in acid fractions, corresponding to the leukotactic activity in these same fractions.

The results of experiments employing trypsinized and nontrypsinized leukotactic fractions from activated serum were similar to those described above (Text-figure 4). Maximal peaks of absorbance at 280 nm were located in fractions corresponding to pH 3 and pH 4 through 6. A major peak of leukotactic activity was found in the undigested (but not the trypsinized) samples. This activity was found in fractions between pH 7 and pH 9. Tumor cell chemotactic activity generated in parallel samples



TEXT-FIGURE 4—Isoelectric focusing of leukotactic fractions of zymosan-activated serum. Leukotactic fractions of zymosan-activated serum were applied to an isoelectric focusing column. A second sample was applied following trypsin treatment for 30 minutes (1% trypsin [w/v] at 37 C, stopped with 2% soybean trypsin inhibitor). Fractions were assayed for pH, absorbance at OD_{280}, chemotactic activity for tumor cells, and chemotactic activity for leukocytes.

trypsinized for 30 minutes was located in the most basic fractions of the Sephadex gel.

Thus, by employing techniques that permit separation of peptides based on size and/or charge differences, it was possible to achieve separation of the chemotactic factors for leukocytes and for tumor cells. The behavior on gel filtration and in isoelectric focusing of the tumor cell chemotactic activity was identical when the chemotactic factor was gener-

Table 1—Generation of Chemotactic Activity for Tumor Cells From Leukotactic Factors Isolated by Isoelectric Focusing

Chemotactic factor	Cells migrated/high power field	
	Leukocytes	Tumor cells*
Pooled leukotactic fractions†	76 ± 6	18 ± 1
Pooled leukotactic fractions after trypsinization‡	15 ± 2	34 ± 5
Pooled fractions with tumor cell chemotactic activity§	13 ± 2	34 ± 3
Medium 199	16 ± 3	21 ± 3
E coli culture fluid	123 ± 11	23 ± 2

* Walker carcinosarcoma ascites tumor cells
† Pooled Fractions 9 through 11 from the experiment in the upper frame of Text-figure 4
‡ Aliquot of pooled Fractions 9 through 11 trypsinized for 30 minutes
§ Pooled Fractions 10 through 12 from the experiment in the lower frame of Text-figure 4

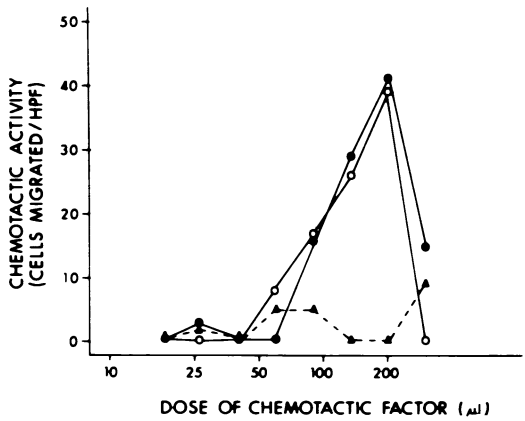
ated either from intact C5 or from the leukotactic activity isolated from activated whole serum.

Generation of Chemotactic Activity for Tumor Cells From Leukotactic Factors Isolated by Isoelectric Focusing

Samples containing leukotactic activity were obtained by isoelectric focusing of leukotactic fractions from zymosan-activated serum. These corresponded to Fractions 9 through 11 in the experiment shown in the upper frame of Text-figure 4. After being assayed for both tumor cell chemotactic activity (which was absent) and leukotactic activity (which was present), the leukotactic fractions were pooled and trypsinized (1% w/v for 30 minutes). Following trypsinization, leukotactic activity was lost in the pool, but chemotactic activity for tumor cells was present (Table 1). This experiment provides the most direct evidence that the tumor cell chemotactic factor is a derivative of the leukotactic factor.

Dose Responses of Different Tumor Cells to the C5 Fragment

For these studies we examined the migration of Walker carcinosarcoma cells (obtained from ascites fluid) (Figure 1), the same tumor maintained in monolayer tissue culture (Figure 2), and the EL-4 murine malignant lymphoma. We first studied the responses of these three cell types under conditions in which the concentration of the stimulus in the lower half of



TEXT-FIGURE 5—Effect on migration of tumor cells of varying the concentration of chemotactic factor in the lower compartment of the Boyden chamber. The dose of chemotactic factor represents the volume of chemotactic factor in 1 ml of Hanks' solution placed in the lower compartment. The chemotactic factor was trypsin-treated leukotactic fractions of zymosan-activated serum. The background migration (when Hanks' solution alone was placed in the lower compartment) for the three cell types was: Walker ascites tumor (*solid circles*), 15 cells/high power field; cultured Walker tumor (*open circles*), 23 cells/high power field; and EL-4 malignant lymphoma (*triangles*), 33 cells/high power field.

Table 2—Checkerboard Analysis of the Migratory Response of Walker Ascites Tumor Cells

Dose of factor in the upper compartment (μl)†	Tumor cell migration/high power field* Dose of factor in the lower compartment (μl)‡			
	12.5	25	50	100
12.5	22 ± 2	40 ± 5	57 ± 7	71 ± 5
25	33 ± 2	39 ± 5	60 ± 6	27 ± 3
50	20 ± 2	25 ± 3	36 ± 2	18 ± 2
100	22 ± 2	17 ± 2	19 ± 2	8 ± 1

* Number of cells migrating in the absence of a chemotactic factor above or below the filter was 11 ± 1/hpf.

† Tumor cells suspended in medium 199 with 10% fetal calf serum at a concentration of 4×10^6 cells/ml. Each milliliter of this suspension contained the designated volume of trypsin-treated leukotactic fractions of zymosan-activated serum.

‡ Volume of leukotactic fractions of zymosan-activated serum in 1 ml of Hanks' medium

the Boyden chamber was varied. The checkerboard analysis of Zigmond and Hirsch [12] was used to determine if the migrational responses were truly chemotactic rather than chemokinetic.

Serial dilutions of trypsinized (30-minute digestion) leukotactic fractions from zymosan-activated serum were prepared over a range of concentrations varying from 300 μl substrate/ml to 18 μl substrate/ml. When the migratory responses of the two Walker tumor cell lines were plotted against the logarithm of the concentration of the stimulating factor, a sigmoidal dose-response curve was obtained (Text-figure 5). When the concentration of stimulant was increased above that which caused maximum migration, fewer cells migrated into the filters. Although varying the dose of stimulating factor had a similar effect on the migration of the two Walker tumor lines, there was little if any appreciable effect on the migration of EL-4 cells. The apparent lack of migration-enhancing activity for the EL-4 cells was confirmed by varying the concentration of the stimulant over a range from 1000 μl/ml (undiluted) to 1 μl/ml.

Checkerboard (Zigmond-Hirsch) Titration of C5 Chemotactic Factor for Tumor Cells

By systematically varying the concentration of trypsinized leukotactic fractions from zymosan-activated serum on both sides of the micropore filters (Tables 2 and 3), it was possible to analyze the effect on cell migration of various concentration gradients.

These experiments demonstrated that optimum enhancement of cell migration occurred when the concentration of trypsinized leukotactic fractions of zymosan-activated serum was higher in the lower well of the chamber than in the upper well. Some stimulation of migration was also observed when the concentration was increased in the absence of a gradient across the micropore membrane. In experiments with both cul-

Table 3—Checkerboard Analysis of the Migratory Response of Cultured Walker Tumor Cells

Tumor cell migration/high power field*				
Dose of factor in the upper compartment (μl)†	Dose of factor in the lower compartment (μl)‡			
	25	50	100	200
25	17 ± 1	33 ± 3	36 ± 3	58 ± 4
50	25 ± 2	32 ± 2	36 ± 4	37 ± 2
100	25 ± 2	28 ± 2	31 ± 2	30 ± 2
200	31 ± 2	31 ± 3	28 ± 2	26 ± 1

* Number of cells migrating in the absence of a chemotactic factor above or below the filter was 22 ± 1/hpf.

† Tumor cells suspended in medium 199 with 10% fetal calf serum at a concentration of 5×10^5 cells/ml. Each milliliter of this suspension contained the designated volume of trypsin-treated leukotactic fractions of zymosan-activated serum.

‡ Volume of leukotactic fractions of zymosan-activated serum in 1 ml of Hanks' medium

tured cells and ascites tumor cells, the number of cells migrating into the filters decreased at high concentrations of stimulant in the absence of a gradient. The findings indicate that the response of tumor cells to the C5 peptide is chemotactic but that an effect (chemokinesis) on random migration is also detectable. Essentially the same phenomenon has been described for the interaction of a number of leukotactic factors with neutrophils.[12,15]

Discussion

We have shown that chemotactic factors for tumor cells and for leukocytes can be generated from the intact fifth component of complement or from partially purified leukotactic fractions of zymosan-activated serum. The leukotactic and tumor cell chemotactic activities can be separated by physicochemical means. There is evidence that the tumor cell chemotactic activity may be a derivative of the factor responsible for the leukotactic activity.

This work and that of Romualdez et al [3,5,6] ascribe a heretofore unrecognized biologic function to the complement proteins, specifically a chemotactic activity for neoplastic cells. Our work indicates that this function is distinct from the chemotactic activity for leukocytes: the two activities are generated at different times following the initiation of proteolysis of C5; the generation of tumor cell chemotactic activity is accompanied by a decline in leukotactic activity; separation of the two activities can be accomplished by gel filtration or isoelectric focusing. Gel filtration suggests that the factor responsible for tumor cell chemotactic activity is smaller than that responsible for leukotactic activity. While separation of the two activities can also be achieved by isoelectric focusing, the fact that the activity associates in the extreme alkaline range of the pH gradient

suggests that the estimated isoelectric point may be unreliable. However, the procedure does provide evidence that the leukotactic and tumor cell chemotactic peptides can be separated.

The tumor cell chemotactic activities derived from either C5 or from leukotactic fractions of activated serum have similar biologic and physicochemical characteristics as judged by their behavior in gel filtration and isoelectric focusing and by their inactivation after treatment with antibodies to C5. The latter provides the best evidence that the activity derived from activated serum is related to C5 and, together with the former observations, suggests that the C5-derived and activated serum-derived activities are the functions of similar or identical peptides.

A number of experiments suggest that the C5-associated tumor cell chemotactic factor is a derivative of the C5-associated leukotactic peptide. Tumor cell chemotactic activity can be generated from leukotactic products which have been partially purified from trypsinized C5 [6] or from leukotactic fractions of activated serum which have been isolated by isoelectric focusing (Table 1). A natural inhibitor of C5-related leukotactic activity, the α-globulin chemotactic factor inactivator,[16] inactivates leukotactic activity and generates tumor cell chemotactic activity from activated serum or partially trypsinized C5. The tumor cell chemotactic factor appears to be smaller than the leukotactic factor based on studies in gel filtration (Text-figure 2) and ultracentrifugation.[3] This size difference is also consistent with the concept that the former is a derivative of the latter. A definitive statement concerning the derivation of the tumor cell chemotactic factor from the leukotactic factor awaits complete isolation and chemical characterization of the two factors.

The data presented herein represent the first direct evidence that malignant neoplastic cells are capable of motility responses that are dose-dependent and truly chemotactic. Thus, it has been shown that tumor cells, like leukocytes, may respond to the presence of chemical stimuli by both enhanced random migration as well as directed movement, the latter occurring when the cells are exposed to a concentration gradient of the stimulus. In the absence of a gradient, random migration of tumor cells is enhanced by the chemotactic factor in amounts that elicit chemotactic responses in a concentration gradient. As observed in studies of leukotaxis, there is inhibition of both enhanced random migration and chemotactic responses if the concentration of the chemotactin is increased beyond that which elicits a maximum response of the cells.[16]

In addition to showing that ascites tumor cells from tumor-bearing animals are capable of chemotactic movement, we have also shown that cultured tumor cells are capable of chemotactic movement. The potential usefulness of tissue culture as a source of cells for evaluating their chemotactic responses and related biologic phenomena is indicated by this

study and by two recent reports concerning the chemotactic responses of cultured macrophages.[17,18] We believe that tissue culture may be of considerable value by providing access to populations of tumor cells of widely differing biologic behavior and histogenesis.

An example of this application is also provided; this report documents, for the first time, a distinct difference in the responses of two malignant neoplastic cell lines to the complement-related chemotactic factor for tumor cells. Although both Walker carcinosarcoma lines responded to the chemotactin in a dose-dependent manner, cells from the EL-4 malignant lymphoma migrated readily into the micropore filters but failed to demonstrate alteration of migration in response to varying doses of the chemical stimulus.

Such variation in the responses of cells of different derivation might be expected since there is evidence that chemotactically responsive cells of different types demonstrate considerable selectivity in their reaction to various chemoattractants. Such specificity is actually illustrated in Text-figure 1, in that, as chemotactic activity for tumor cells is generated, there is a parallel decline in leukotactic activity.

We are only beginning to elucidate the role of the chemotactic response of tumor cells in the process of metastasis. The studies of Hayashi and Ozaki, employing a tissue-derived chemotactic factor, clearly indicate that *in vivo* the chemotactic responses of tumor cells may be one mechanism by which circulating tumor cells emigrate from the bloodstream and establish secondary tumor sites.[1,4] The process is analogous to the mechanism by which leukocytes migrate into an inflammatory focus. In terms of this analogy, both random migration and chemotaxis might be means by which tumor cells set up secondary foci. We have recently shown, for example, that at least one tumor, a murine fibrosarcoma, is partially composed of a small subpopulation of cells which have unique random migratory characteristics, independent of the major population of cells, and which are absent from normal control fibroblast populations.[19] In the present study we have shown that at least some tumor cells, like leukocytes, are capable of both enhanced random migration and chemotactic responses to a complement-derived chemotactic factor.

In addition to the C5-derived chemotactic factor for tumor cells, the existence of at least one and possibly two other tumor cell chemotactic factors has been shown by Ushijima et al.[20] This suggests the possibility that, as in the case of leukocytes, there may be multiple chemotactic factors for tumor cells with a variety of cell specificities. If so, the established patterns of metastatic growth, which are characteristic of various tumors, may be the consequence of the responses of specific tumor cell types to chemotactic factors generated by different means in various tissues.

References

1. Hayashi H, Yoshida K, Ozaki T, Ushijima K: Chemotactic factor associated with invasion of cancer cells. Nature 226:174–175, 1970
2. Yoshida K, Ozaki T, Ushijima K, Hayashi H: Studies on the mechanisms of invasion in cancer. I. Isolation and purification of a factor chemotactic for cancer cells. Int J Cancer 6:123–132, 1970
3. Romualdez AG Jr, Ward PA: A unique complement derived chemotactic factor for tumor cells. Proc Natl Acad Sci USA 172:4128–4132, 1975
4. Ozaki T, Yoshida K, Ushijima K, Hayashi H: Studies on the mechanisms of invasion in cancer. II. *In vivo* effects of a factor chemotactic for cancer cells. Int J Cancer 7:93–100, 1971
5. Romualdez AG Jr, Ward PA: Further studies on the C5-derived chemotactic factor for tumor cells. Membranes and Neoplasia—New Approaches and Strategies: Proceedings of a Conference Held March 3–6, 1976, in Keystone, Colorado. (Progress in Clinical and Biological Research: Vol 9.) Edited by VT Marchesi. New York, Alan R. Liss, Inc., 1976, pp 65–71
6. Romaldez AG Jr, Ward PA, Torikata T: Relationship between the C5 peptides chemotactic for leukocytes and tumor cells. J Immunol 117:1762–1766, 1976
7. Cohn ZA, Morse SI: Interactions between rabbit polymorphonuclear leucocytes and staphylococci. J Exp Med 110:419–443, 1959
8. Nilsson UR, Tomar RH, Taylor FB Jr: Additional studies on human C5: Development of a modified purification method and characterization of the purified product by polyacrylamide gel electrophoresis. Immunochemistry 9:709–723, 1972
9. Vallota EH, Müller-Eberhard HJ: Formation of C3a and C5a anaphylatoxins in whole human serum after inhibition of the anaphylatoxin inactivator. J Exp Med 137:1109–1123, 1973
10. Ward PA, Lepow IH, Newman LJ: Bacterial factors chemotactic for polymorphonuclear leukocytes. Am J Pathol 52:725–736, 1968
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951
12. Zigmond SH, Hirsch JG: Leukocyte locomotion and chemotaxis: New methods for evaluation, and demonstration of a cell-derived chemotactic factor. J Exp Med 137:387–410, 1973
13. Radola BJ: Isoelectric focusing in layers of granulated gels. II. Preparative isoelectric focusing. Biochim Biophys Acta 386:181–195, 1975
14. Andrews P: Estimation of the molecular weights of proteins by Sephadex gel-filtration. Biochem J 91:222–233, 1964
15. Wilkinson PC, McKay IC: Recognition in leucocyte chemotaxis: Studies with structurally modified proteins. Antibiot Chemother 19:421–441, 1974
16. Till G, Ward PA: Two distinct chemotactic factor inactivators in human serum. J Immunol 114:843–847, 1975
17. Snyderman R, Pike MC, Fischer DG, Koren HS: Biologic and biochemical activities of continuous macrophage cell lines P388D1 and J774.1. J Immunol 119:2060–2066, 1977
18. Gallin EK, Gallin JI: Interaction of chemotactic factors with human macrophages: Induction of transmembrane potential changes. J Cell Biol 75:277–289, 1977
19. Varani J, Orr W, Ward PA: A comparison of the migration patterns of normal and malignant cells in two assay systems. Am J Pathol 90:159–172, 1978
20. Ushijima K, Nishi H, Ishikura A, Hayashi H: Characterization of two different factors chemotactic for cancer cells from tumor tissue. Virchows Archiv [Cell Pathol] 21:119–131, 1976

Acknowledgments

The authors would like to thank Mr. Tim Flynn, Ms. Sandy Goralnick, and Ms. Patricia Perone for their technical assistance with this work.

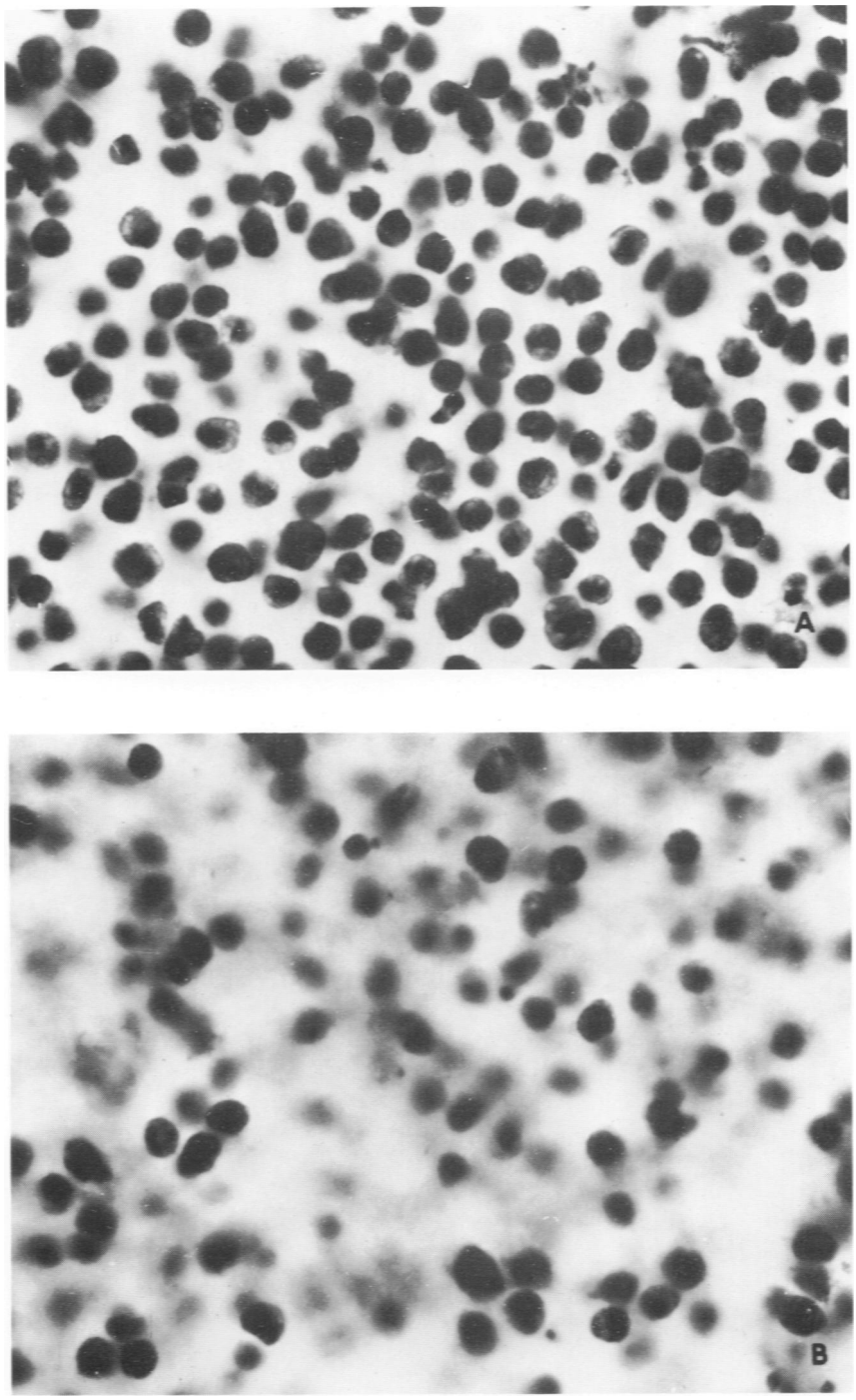


Figure 1A—Walker carcinosarcoma ascites tumor cells attached to the upper surface of the micropore membrane. **B**—Walker carcinosarcoma cells that have migrated 25 μ into the interstices of the micropore membrane. Approximately 40 cells are located at this level in this photograph. (Hematoxylin, × 400)

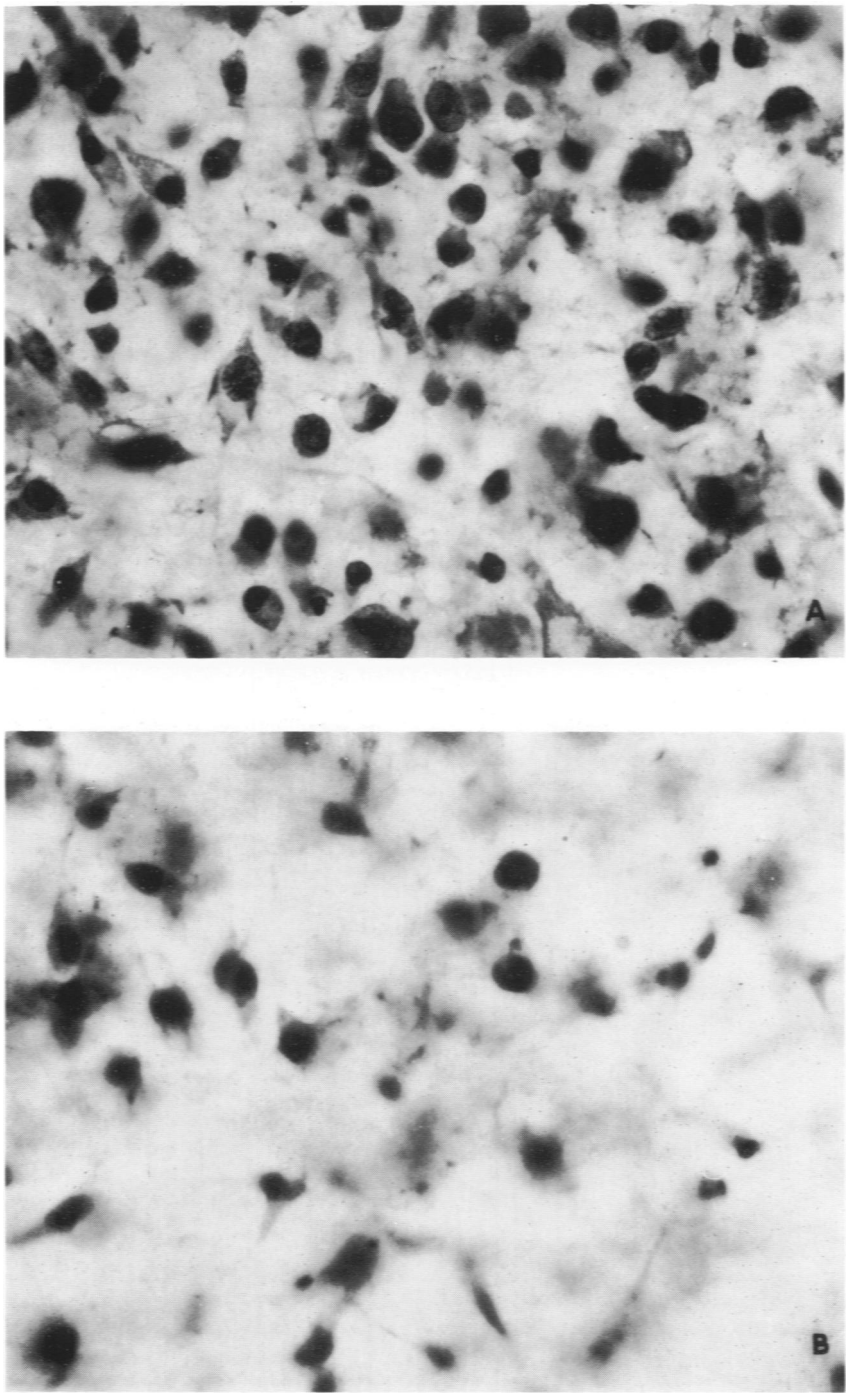


Figure 2A—Walker carcinosarcoma cells from tissue culture attached to the upper surface of the micropore membrane. **B**—Walker carcinosarcoma cells from tissue culture that have migrated 25 μ into the interstices of the micropore membrane. Approximately 20 cells would be counted in this photograph. (Hematoxylin, × 400)