Detection of a Complement-Derived Chemotactic Factor for Tumor Cells in Human Inflammatory and Neoplastic Effusions

F. W. ORR, MD, E. J. DELIKATNY, BSc, S. MOKASHI, PhD, G. V. KREPART, MD, and H. G. STIVER, MD

From the Departments of Pathology, Obstetrics, Gynecology and Reproductive Science, and Internal Medicine, University of Manitoba, Winnipeg, Manitoba, Canada

A chemotactic factor for neoplastic cells can be generated *in vitro* by incubating human C5 or C5a with leukocytic or pancreatic lysosomal enzymes and is also detectable in experimental inflammatory exudates. The authors therefore sought evidence for the existence of this factor in human effusions. Using the Boyden chamber assay, they detected chemotactic activity for MB-MDA-231 human breast carcinoma cells and Walker ascites tumor cells in human inflammatory and neoplastic exudates, including ascites, pleural effusions, synovial fluids and cerebrospinal fluids. Chemotactic activity was not found in transudates, normal cerebrospinal fluid, or normal serum. Human ovarian adenocarcinoma cells from one of the effusions migrated toward autologous ascites and towards the C5-derived chemotactic factor that had been prepared *in vitro*. In gel filtration the chemotactic factor behaved generally as a molecule having a molecular weight of ∿6000 daltons. The activity was blocked after incubation with antiserums directed against C5 but not by antiserums directed against C3 or C4. *In vitro*, chemotactic activity for tumor cells could be generated by incubating extracts of exudate cells with autologous plasma or with purified C5. The authors conclude that a chemotactic factor for tumor cells can be formed in human effusions and that this factor has properties similar to those of a previously described C5-derived chemotactic factor. (Am J Pathol 1983, 110:41–47)

IN COMPLEX ORGANISMS, such as man, the chemotaxis of freely moving cells is important in several processes, including embryogenesis, acute inflammation, and wound healing.[1] Similarly, recent data indicate that the chemotactic responses of malignant tumor cells can contribute to the localization and metastasis of circulating tumor cells.[2–5] A question deriving from these observations concerns how chemoattractants for tumor cells are generated. Regarding one factor, controlled enzymatic digestion of purified C5 and C5a *in vitro* results in the formation of an agent that is biologically active as a chemoattractant for cells from several different tumor lines.[6,7] The enzymes demonstrated to be capable of effecting this generation include trypsin, crude prepartions of human leukocyte lysosomal granules, phagocytic supernatant fluids from human peripheral neutrophils, and the purified leukocyte neutral proteases elastase and cathepsin G.[8] Since lysosomal enzymes and the substrates C5 and C5a are likely to be found in inflammatory exudates,[9] we postulated that such fluids might provide an appropriate environment for the generation of the C5-derived chemotactic factor for tumor cells. Thus, we have recently demonstrated that the factor can be detected in experimentally induced inflammatory exudate fluids and that it can promote the local arrest and metastasis of circulating tumor cells.[10]

Here we demonstrate that the C5-related chemotactic factor for tumor cells can also be identified in human exudate fluids and that it is biologically active as a chemoattractant for human tumor cells.

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Dr. Orr is a Clinical Research Associate of the National Cancer institute of Canada.

Dr. Stiver's present address is Department of Medicine, University of British Columbia, Vancouver, Canada.

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Address reprint requests to F. William Orr, MD, Department of Pathology, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3 Canada.

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Materials and Methods

Exudates and Control Fluids

The specimens we examined consisted of aliquots of ascites fluids, pleural and synovial effusions, and cerebrospinal fluids collected for diagnostic or therapeutic purposes from patients at the Health Sciences Centre, Winnipeg, Manitoba, Canada. Immediately following collection, the fluids were heparinized (10 IU heparin/ml), a total cell count was performed, and the samples were centrifuged at 250*g* for 10 minutes. A differential count was performed on a smear prepared from the cell pellet. The supernatant fluids were analyzed for total protein content.[11] We determined the concentration of C5 by radial immunodiffusion[12] using commercially prepared reagents (Meloy Laboratories, Springfield, Va). We measured the lysosomal enzyme β-N-acetylglucosaminidase according to the method of Bossmann et al,[13] employing *p*-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate. We measured β-glucuronidase by the method of Musa et al,[14] employing phenolphthalein mono-β-glucuronic acid as a substrate. We measured lactic dehydrogenase by the method of Wroblewski and LaDue,[15] employing reduced diphosphonucleotide (DPNH) and pyruvic acid (sodium salt) as substrates. All substrates for these analyses were purchased from Sigma Chemical Co., St. Louis, Missouri. Data from these analyses are presented in Table 1.

Tumor Cells

Walker carcinosarcoma ascites tumor cells, which are responsive to the human C5-derived chemotactic factor for tumor cells, were used as the reference cell line throughout this study. The maintenance of these cells in Sprague-Dawley rats and their preparation for chemotaxis assays has been described previously.[16]

MD-MDA-231 cells are an established human breast carcinoma line, originally from the pleural effusion of a patient with a metastatic tumor.[17] These were provided by Dr. Gregory Mundy. They were maintained by serial passage in tissue culture and prepared for chemotaxis studies by methods described previously for cultured cells.[16]

Human tumor cells were also obtained from cell pellets after centrifugation of neoplastic effusions. Following osmotic lysis of erythrocytes, the cells were resuspended and washed 3 times in tissue culture medium 199. We then suspended the cells at a concentration of 1×10^6 cells/ml in tissue culture flasks and incubated them at 37 C in 5% CO_2 for 2 hours in order to allow leukocytes to adhere to the plastic surface of the flasks. The relatively nonadhesive tumor cells were then poured off and washed again in medium 199. Differential counts were done with a Giemsa stain, and cellular viability was determined by means of trypan blue exclusion. For chemotaxis experiments the cells were then suspended at a concentration of 5×10^6 viable tumor cells/ml in medium 199 containing 5% fetal calf serum.

Determination of Chemotactic Activity

Analysis of cell-free fluids was performed immediately or after storage at −20 C. Chemotaxis assays were performed by the Boyden chamber–micropore filter technique as described in detail previously.[16] Serial dilutions of the various test fluids were prepared in medium 199 and placed into the lower compartment of the Boyden chambers. From dose-response data obtained in these experiments it was possible to determine the effective dose range for each effusion and the dose that elicited a half-maximum chemotactic response (ED_{50}). The chemotactic activity in each effusion was expressed as the number of ED_{50}/ml. In each experiment a positive control consisted of the C5-derived chemotactic factor for tumor cells prepared by trypsinization of partially purified C5a.[16] We determined values for random migration by placing culture medium alone into the lower well of the Boyden chamber. Statistical analysis was performed by means of the Student *t* test.

Gel Filtration Chromatography of Exudate Supernatant Fluids

A calibrated column of Sephadex G75 (Pharmacia, Piscataway, NJ), measuring 60 cm × 1.6 cm (bed volume of 120 ml) was eluted with Hanks' balanced salt solution, pH 7.4. The calibration standards were blue dextran (mol wt 2×10^6 daltons), chymotrypsinogen A (mol wt 2.5×10^4 daltons), myoglobin (mol wt 17.8×10^3 daltons), cytochrome C (mol wt 12.4×10^3 daltons), insulin (mol wt 6×10^3 daltons), and phenol red (mol wt 3.5×10^2 daltons) (Sigma Chemical Co., St. Louis, Mo). One milliliter of each effusion in 4 ml Hanks' solution was applied to the column. Fractions of 5 ml were collected. In order to assay the fractions for the presence of chemotactic activity, we diluted 500 μl of each fraction with medium 199 to a final volume of 1 ml and injected it into the lower compartment of the Boyden chamber.

Antibody Blocking Experiments

Goat antiserums to the human complement components C3, C4, and C5, and antiserums against whole

Table 1—Source and Characteristics of Specimens Examined for Chemotactic Activity

Diagnosis	Source of specimen*	n	Protein content: Total (g/dl)	Protein content: C5 (mg/dl)	Enzymatic activities: Glucosaminidase†	Enzymatic activities: β-Glucuronidase‡	Enzymatic activities: LDH§	Cellular content: Neutrophils/ml	Cellular content: Other cells/ml¶
Peritonitis pleuritis	A/PE	3	3.4 ± 1.7	6.3 ± 3.6	42.7 ± 17.8	1.4 ± 0.5	37.8 ± 3.9	$9.0 \pm 2.0 \times 10^4$	$5.4 \pm 0.9 \times 10^5$
Meningitis	CSF	2	3.7 ± 2.5	—	—	—	—	$5.6 \pm 1.0 \times 10^5$	0
Rheumatoid arthritis	SF	3	4.3 ± 0.2	—	—	—	99.2 ± 26.7	$2.3 \pm 1.1 \times 10^7$	$1.5 \pm 0.4 \times 10^6$
Metastatic carcinoma	A/PE	11	4.0 ± 0.2	7.9 ± 0.6	46.5 ± 9.0	2.5 ± 0.3	50.0 ± 9.0	$2.3 \pm 1.2 \times 10^6$	$6.3 \pm 4.0 \times 10^6$**
Congestive heart failure, cirrhosis	A/PE	9	3.3 ± 0.4	4.6 ± 0.4	16.8 ± 2.9	0.7 ± 0.2	11.2 ± 2.5	$4.0 \pm 1.0 \times 10^4$	$2.6 \pm 0.5 \times 10^5$
Normal	CSF	6	$3.6 \pm 0.9 \times 10^{-2}$	0	—	—	—	0	$1.7 \pm 0.6 \times 10^2$
Normal	Serum	4	7.0 ± 0.6	10.3 ± 0.2	29.0 ± 3.0	1.7 ± 0.2	18.5 ± 2.4	0	0

* A, ascites; PE, pleural effusion; CSF, cerebrospinal fluid; SF, synovial fluid.
† mmoles phenol liberated/30 min/dl at 37 C.
‡ mmoles phenolphthalein liberated/hour/dl at 37 C.
§ IU activity/dl.
¶ Lymphocytes, monocytes and mesothelial cells, or ependymal cells.
** Neoplastic exudates also contained an average of $1.2 \pm 0.4 \times 10^6$ tumor cells/ml.

human serum were purchased from Calbiochem-Behring Corp., La Jolla, California. The antigenic specificity of these reagents was confirmed by immunodiffusion as described previously.[16] Before use these anitserums were inactivated by heating at 56 C for 30 minutes. In experiments designed to test the suppressive effects of the antiserums on chemotactic activity in the effusions, 25-μl volumes of antiserum or normal goat serum were incubated at 37 C for 30 minutes with 1 ml of medium containing a volume of each effusion sufficient to elicit a chemotactic response of the tumor cells. The antibody-treated effusions were then assayed in Boyden chambers. In each experiment additional controls consisted of an equal volume of the effusion incubated for 30 minutes in the absence of antiserum, and 25-μl aliquots of the various antiserums in medium 199 without the effusion.

Generation of Chemotactic Activity *in Vitro*

The purified fifth component of complement was prepared from normal human serum according to the method of Nilsson et al.[18] The purity of the material obtained was tested by immunodiffusion and by electrophoresis in polyacrylamide gels.[16] We prepared extracts of exudate cells or dextran-sedimented peripheral blood leukocytes by subjecting cell pellets (0.5 g wet tissue/ml medium 199) to four cycles of freeze–thawing and subsequent centrifugation to remove insoluble contents. Purified C5 was treated with 1% trypsin (wt/wt) or with 1 μl cell extract/10 μg C5 for 60 minutes at 37 C in an agitating water bath. Soybean trypsin inhibitor (2% wt/wt) was then added to samples treated with trypsin. Equal volumes of plasma and autologous cell extract were incubated at 37C for 60 minutes. Serial dilutions were prepared of plasma alone, cell extract alone, and the plasma-cell extract incubation mixture. These were tested for chemotactic activity.

Results

Presence of Chemotactic Activity for Tumor Cells in Exudate Fluids

Chemotactic activity for tumor cells was found in all of the inflammatory and neoplastic exudates but was not found in the transudates, in normal cerebrospinal fluid, or in normal serum (Table 2). Sigmoidal dose-response curves were obtained over the effective dose range of each fluid. In all cases the dose of fluid that elicited a half-maximum chemotactic response (ED_{50}) was obtained by our diluting the fluid many times. The migration of cells observed at the points of maximum response was in all cases significantly greater than the random migration of the same cell populations ($P < 0.025$).

One patient had an infected ventriculoatrial shunt. In this patient, examination of the cerebrospinal fluid obtained at the time of diagnosis demonstrated *Staphylococcus aureus* (phage type 83a/81) by culture, and the fluid contained chemotactic activity for tumor cells (83 ED_{50} units/ml). *S aureus* was not cultured from a second sample obtained after a 9-day period

Table 2—Chemotactic Activity for Tumor Cells in Human Exudates

Diagnosis	Walker Carcinosarcoma Number positive	Walker Carcinosarcoma Activity*	MB-MDA-231 cells Number positive	MB-MDA-231 cells Activity*
Peritonitis/pleuritis	3/3	543 ± 255	1/1	550
Meningitis	3/3	51 ± 24	—	—
Rheumatoid arthritis	3/3	429 ± 90	—	—
Metastatic carcinoma	11/11	533 ± 103	9/10	270 ± 84
Congestive heart failure, cirrhosis	0/9	0	0/9	0
Normal CSF	0/6	0	—	—
Normal serum	0/4	0	0/3	0

* Mean number of ED_{50} units of biologic activity/ml ± SEM.

of treatment with cloxacillin and rifampin, and the chemotactic activity had decreased (4 ED_{50}/ml). Microorganisms were not recovered from a third sample of cerebrospinal fluid taken after 14 days of antibiotic therapy, and chemotactic activity was not detected. (data not shown).

We were able to examine the migration of tumor cells obtained from the neoplastic exudate obtained from a patient with extensive peritoneal and pleural involvement by a poorly differentiated adenocarcinoma of ovarian origin. A population consisting of >80% single tumor cells (>95% viability by trypan blue exclusion) was isolated from this patient's ascites. These cells were tested immediately for chemotactic responsiveness in the Boyden chamber assay, using serial dilutions of autologous ascites supernatant fluid as a presumptive chemoattractant. The patient's own cells, the MB-MDA-231 cells, and the Walker ascites cells showed dose-dependent responses to the exudate (Figure 1). All three cell types also responded to the C5-related chemotactic factor over a similar dose range (data not shown). Movement of cells was greatest when the attractant was present on the opposite side of the micropore filter, but enhanced migration was also observed when the concentration of the stimulant was equal on both sides. Very slight stimulation of movement was seen when the cells were suspended in the attractant and when medium was present on the opposite side of the filter (Table 3).

Analysis of Chemotactic Activity

Each exudate containing chemotactic activity was chromatographed on a calibrated column of Sephadex G75 in order to compare the behavior of the chemotactic factor for tumor cells contained therein with the behavior of the factor obtained by proteolytic digestion of purified C5 or C5a *in vitro*. Previous studies have shown that the latter elutes primarily in fractions corresponding to a molecular weight of ~6000 daltons.[7,16] Four pools of chemotactic activity were found (Table 4). The major peak of activity found in all effusions was located in fractions corresponding to a molecular weight of ~6000 daltons. In all but two experiments, fractions eluting in this zone stimulated the greatest amount of chemotactic migration of the tumor cells. In 12 of 15 experiments a second pool of activity was associated with the fractions excluded from the column (void volume, mol wt ≥70,000). In 9 effusions a smaller pool of chemotactic activity was found in fractions eluting just after the cytochrome C marker (mol wt 12.4 × 10^3 daltons). In 2 cases residual activity was found in fractions eluting after the exclusion limits of the column (mol wt <3000).

Previous experiments indicated that antiserums to C5 are capable of inhibiting the chemotactic activity for tumor cells, generated *in vitro* from whole serum[19]

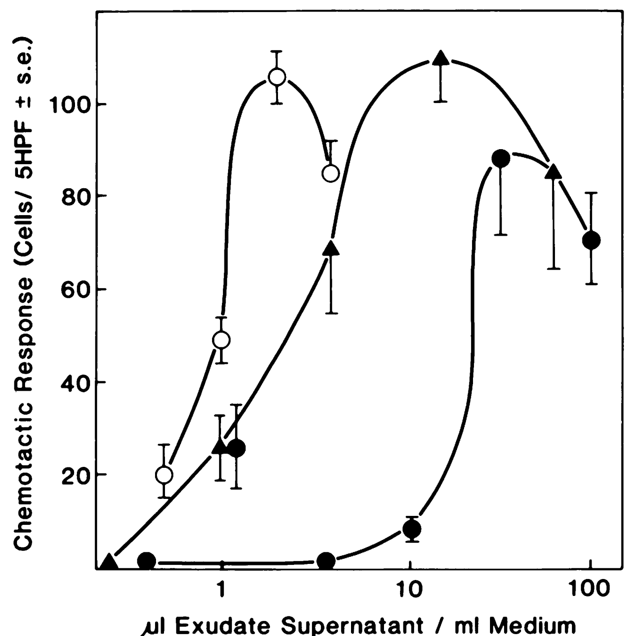

Figure 1—Migration of human ovarian adenocarcinoma cells (●——●), MB-MDA-231 human breast carcinoma cells (▲——▲), and Walker ascites tumor cells (○——○) toward varying concentrations of human exudate supernatant. The ovarian adenocarcinoma cells and the exudate supernatant were from the same patient. Values for random migration have been subtracted.

Table 3—Evidence That the Migration of Tumor Cells in Response to Human Exudates Is Chemotactic in Character

Fluid in upper compartment	Fluid in lower compartment	Cell migration/5 hpf ± SEM† MB-MDA-231 cells Experiment 1	MB-MDA-231 cells Experiment 2	Walker tumor Experiment 3
Medium	Effusion*	69 ± 7	82 ± 15	213 ± 18
Effusion*	Effusion	23 ± 5	25 ± 4	100 ± 14
Effusion*	Medium	8 ± 1	13 ± 7	86 ± 10
Medium	C5-related factor‡	42 ± 8	107 ± 5	188 ± 16
C5-related factor	C5-related factor	14 ± 9	12 ± 5	126 ± 10
C5-related factor	Medium	10 ± 8	0	94 ± 14

* In each of the three experiments 4 effusions were tested separately. On the basis of data summarized in Table 2, these had been diluted in medium 199 so that the final concentration represented 2 ED_{50} units of biologic activity/ml.

† Values are the mean of results on the 4 separate effusions. For each, 10 microscopic fields were examined in duplicate assays. Values for random migration (medium 199 in both compartments) have been subtracted. These were: Experiment 1—71 ± 5 cells/5 hpf; Experiment 2—114 ± 4 cells/5 hpf; Experiment 3—59 ± 12 cells/5 hpf.

‡ C5-related chemotactic factor prepared by digestion of C5a with trypsin as described in Orr et al.[16]

or from purified C5.[8] We wished to determine whether a similar blocking effect could be detected when the effusions were incubated with anti-C5 prior to assaying them for chemotactic activity. In these experiments we found that the chemotactic activity in all effusions was blocked when they were preincubated with serums containing antibodies to C5 or antibodies to whole normal human serum. With the exception of one specimen of cerebrospinal fluid, this effect was not observed when the effusions were incubated with antiserums to C3 or C4 (Table 5). These findings corroborate our previous work and suggest that the chemotactic factor generated *in vivo* shares antigenic determinants in common with C5.

Generation of Chemotactic Activity *in Vitro*

The purpose of these experiments was to extend the previous observation that the chemotactic factor for tumor cells can be generated by digestion of purified C5 with enzymes obtained from peripheral blood leukocytes.[8] We wished to determine whether chemotactic activity for tumor cells could be generated by an interaction between exudate cells and autologous plasma, representing *in vitro* a potential interaction between the humoral and cellular constituents of exudate fluids. Human plasma, extracts of exudate cells, and intact C5 lacked chemotactic activity. However, chemotactic activity was generated by incubating extracts of exudate cells or leukocytes with purified human C5 or with autologous plasma (Table 6). Chemotactic activity was not observed following incubation of intact exudate cells or leukocytes with autologous plasma (data not shown).

Table 4—Chromatographic Analysis of Human Effusions on Sephadex G75

Fraction	Molecular weight	Number positive	Percentage of total chemotactic activity*
I	$>7 \times 10^4$	12/15	20 ± 5
II	$11 \pm 2 \times 10^3$	9/15	10 ± 4
III	$6 \pm 1 \times 10^3$	15/15	69 ± 6
IV	$<3 \times 10^3$	2/15	1 ± 1

* Values represent the percentage of total observed chemotactic activity in positive effusions.

Discussion

The data presented here demonstrate that a chemotactic factor for human and animal tumor cells can be detected in human exudate fluids. These results extend our observations made in experimental animals[10] and support the hypothesis that *in vivo*, as *in vitro*, the generation of a C5-related chemotactic activity for neoplastic cells is a potential result of interactions between the cellular and humoral components of exudate fluids. We postulate that cellular degradation or stimulation of neutrophils in the inflammatory reaction, leading to release of proteolytic enzymes,[20,21] could, in turn, degrade C5 or C5a to form the chemotactic factor for tumor cells.[8] Our analyses of exudates and control fluids indicate that C5 and the cellular components necessary for such an interaction are likely to be present in exudate fluids (Tables 1 and 6).

The chemotactic factor generated *in vivo* has properties like those of the factor that can be generated *in vitro* by proteolysis of C5 of C5a.[7,16] In gel filtration most of the chemotactic activity was located in fractions corresponding to a molecular weight of $6 \pm 1 \times 10^3$ daltons. The observation that a smaller proportion of the total chemotactic activity was located in fractions eluting with the void volume and fractions with a mean molecular weight of $11 \pm 2 \times 10^3$

Table 5—Inhibition of Chemotactic Activity in Human Effusions by Antiserums Against Complement Proteins

Antiserum*	Number inhibited	% Inhibition of total activity†
Anti-C3	7/15	16 ± 7
Anti-C4	2/15	1 ± 1
Anti-C5	15/15	94 ± 2
Anti-whole serum	15/15	90 ± 3

* 25 μl of antiserum or normal goat serum was incubated at 37 C for 30 minutes with 1 ml of medium containing sufficient effusion to represent 2 ED_{50} units of biologic activity.

† Inhibition of the response obtained after incubation with normal goat serum. (Incubation with normal goat serum inhibited the activity of untreated samples by 35% ± 5%.)

daltons can be compared to the experience of Romualdez and Ward.[6] Using sucrose density gradient analysis as well as gel filtration, they found that the C5-derived chemotactic factor for tumor cells, when generated in whole serum, tends to associate with a factor contained in Cohn Fraction II, possibly an immunoglobulin. However, when generated in agammaglobulinemic serum, activity eluted in two regions of low molecular weight, estimated to be ~14,000 daltons and ~5000 daltons.[6] Likewise, the activity associated with fractions of higher molecular weight can be displaced by chromatography of experimental exudates in 6 M urea.[10] The demonstration that the chemotactic activity in the exudates can be inhibited by incubating the effusions with antiserum to C5 is consistent with the observation that such treatment inhibits the chemotactic activity generated *in vitro* by incubating purified C5 with leukocyte phagocytic supernatant fluids.[8] We have no experimental data to explain a single observation that antiserums to C3 inhibited the chemotactic activity present in one cerebrospinal fluid. In several previous experiments it has not been possible to demonstrate the generation of chemotactic activity for tumor cells by proteolysis of purified C3, although the generation of chemotactic activity for leukocytes was observed.[8,19] Definitive statements concerning the nature of the chemotactic factor will require its isolation and biochemical characterization. This work is in progress.

Migration of the tumor cells was stimulated most when exudate fluid was placed into the Boyden chamber compartment opposite the cells. Lesser degrees of enhanced migration occurred when the exudate fluid was placed into both compartments or when the cells were suspended in the exudate and medium was placed into the opposite compartment. Thus, the responses of the established human and animal lines were both chemotactic and chemokinetic in character.[16,22] This report presents the first evidence that the C5-related chemoattractant can stimulate the motility of human tumor cells. Although the presence of the factor resulted in increased migration of cells from an established breast carcinoma line and cells taken directly from a metastatic ovarian adenocarcinoma, it seems unlikely that all human tumor cells will be found to be responsive to this agent. There are variations in the response patterns of animal tumors, and it is possible that these relate to their degree of differentiation and/or differing malignant potential.[16,23-25] However, before any general statements can be made, it will be necessary to survey the effect of various tumor cell chemoattractants on a broad spectrum of human neoplasms. A major problem in such experiments will be to obtain adequate numbers of single tumor cells, free of other cell types. In this respect, cells from ascites fluids or solid tumors are frequently unsuitable for study in the Boyden chamber because the tumor cells are in aggregates or there are contaminating cell types, eg, leukocytes, mesothelial cells, fibroblasts. Our success in demonstrating a chemotactic response by one human ascites tumor was due largely to the relatively high proportion of single tumor cells in this specimen.

Clinical and experimental observations indicate that some tumors have a tendency to metastasize to sites of tissue injury.[26,27] Mechanisms postulated to explain this phenomenon include the generation of growth-promoting factors at sites of inflammation[28] or activation of coagulation pathways, leading to entrapment of circulating cells.[29] We propose that the generation of chemoattractants at sites of injury and inflammation could promote the arrest and local-

Table 6—Generation of Chemotactic Activity *in Vitro*

Chemoattractant	Chemotactic response (cell migration/5 hpf ± SE)*		
	Experiment A	Experiment B	Experiment C
Human plasma (3.2 μl)	5 ± 3	7 ± 10	8 ± 4
C5 (25 μg)	0	0	—
Exudate cell extract†	1 ± 1	0	2 ± 1
Leukocyte extract†	—	—	0
Human plasma + exudate cell extract‡	83 ± 15	58 ± 8	44 ± 10
Human plasma + leukocyte extract‡	—	—	87 ± 7
C5 + exudate cell extract§	92 ± 13	110 ± 18	—
C5 + trypsin¶	97 ± 17	98 ± 10	—

* Values for random migration have been subtracted.

† Cell extracts were prepared by freeze-thawing the cell pellets obtained after centrifugation of fresh exudates. Peripheral blood leukocytes were obtained by dextran sedimentation and extracts similarly prepared. Cells and plasmas were from the same patients.

‡ Equal volumes of plasma and cell extract were incubated at 37 C for 60 minutes. Serial dilutions were prepared over a wide range and tested for chemotactic activity. Values expressed represent the amount of chemotactic activity generated from 3.2 μl of plasma.

§ 25 μg C5 incubated with 2.5 μl cell extract at 37 C for 60 minutes.

¶ 25 μg C5 digested by 1% trypsin at 37 C for 60 minutes.

ization of circulating, chemotactically responsive human tumor cells, as can be demonstrated in experimental animal systems.[10] Since the chemotactic activity is demonstrable after manyfold dilution of exudate fluids, we argue that a biologically effective concentration gradient could be created *in vivo* across the vessel walls that separate the exudate from the circulation. In addition to directed migration, other cellular responses to chemoattractants could facilitate the arrest and transvascular passage of tumor cells. These phenomena include increased adherence,[4] cellular swelling,[30] and the release or activation of proteolytic enzymes.[31,32]

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