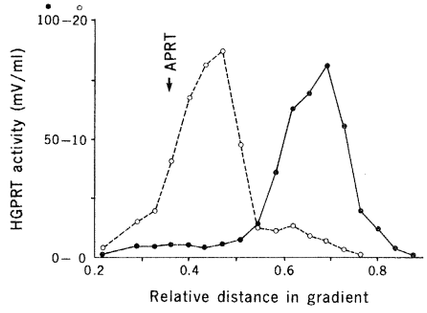

Fig. 4. Sedimentation of HGPRT of human lymphoblasts. Human lymphoblasts (PGLC 33H) (*19*), 10^7 cells per milliliter in 10 m*M* potassium phosphate, *p*H 6.8, 0.25*M* sucrose and 10 m*M* DTT, were lysed four times by freezing and thawing and then sedimented at 100,000*g* for 60 minutes. The supernatant was dialysed overnight at 4°C against the lysis buffer. Samples, containing approximately 70 μg of protein, 0.10 unit of HGPRT, and 0.03 unit of APRT (adenine phosphoribosyltransferase, E.C. 2.4.2.7) in 0.01 ml, were diluted to 0.1 ml in 2 percent ampholyte, *p*H 5 to 7, with 1 m*M* DTT (solvent A) or 1 m*M* potassium phosphate, *p*H 6.8, with 0.25*M* NaCl and 1 m*M* DTT (solvent B). The samples were centrifuged through linear 5 to 20 percent sucrose gradients (5.2 ml) in their respective solvents. Centrifugation was in the SW 50.1 rotor (Beckman) at 50,000 rev/min for 18.5 hours at 2°C. Fractions were collected from the gradient bottom by needle puncture and assayed for HGPRT and APRT. One unit of activity catalyses the conversion of 1 μmole of hypoxanthine (HGPRT) or adenine (APRT) to nucleotide per hour at *p*H 10. One unit of HGPRT activity corresponds to approximately 1 μg of HGPRT protein (*18*). Recoveries of the applied HGPRT and APRT activities in the gradients were 0.016 and 0.006 unit (solvent A) and 0.07 and 0.005 unit (solvent B), respectively. The arrow marks the sedimentation distance of the activity peak observed for the APRT enzyme, which was the same for the two gradients. The molecular weight estimates of HGPRT are based on the relation of sedimentation distance to molecular weight (*20*), and assume a molecular weight of 34,000 for the APRT (*21*). Symbols: ○, HGPRT activity in solvent A; ●, HGPRT activity in solvent B.

sults from modification of the major isoenzyme. On the other hand, the minor enzyme is not detected in the hybrid cells we have analyzed, nor in interspecific hybrids lacking mouse HGPRT (*12*). In the latter hybrids, all of the HGPRT activity is in the major band (data not shown). Therefore, the minor band does not complicate our analysis of heteropolymers in these hybrids.

Because the single HGPRT heteropolymer in hybrids indicates that these enzymes are dimers under the conditions of isoelectric focusing (relatively low ionic strength), and because previous estimates of the molecular weights of the native mouse and human enzymes [68,000 to 100,000 with a subunit size of 26,000 (*5*, *7*)] are substantially greater than that expected for dimers, we have explored the effect of the solvent on the subunit structure of human and mouse HGPRT. In Fig. 4 we compare the results obtained when HGPRT from human lymphoblasts is sedimented in the solvent of isoelectric focusing (2 percent ampholyte) with the results obtained after sedimentation at high ionic strength (0.25*M* NaCl). The sedimentation velocity of the enzyme changes substantially in these two solvents; the molecular weight of HGPRT is estimated to be 48,000 in the ampholyte solvent and 98,000 at high ionic strength, as expected for dimers and tetramers (*13*). These results indicate that, as the ionic strength of the solvent increases, dimers are converted to tetramers (*14*).

Our results demonstrate that human and mouse HGPRT subunits interact to form a single active heteropolymer. The molecular weights obtained for the enzymes sedimented at low ionic strength—the conditions of heteropolymer separation—confirm that the primary structure of the enzyme is a dimer. Furthermore, we have shown that these dimers readily associate to form tetramers at high ionic strength. The equilibrium between these two forms undoubtedly explains why previous estimates of molecular weight have ranged between dimers and tetramers (*4*, *6*).

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13. The slight asymmetry in the activity peak of the enzyme at high ionic strength indicates that a small fraction of HGPRT sediments at a slower rate than that expected for a tetramer. The slower sedimenting species are not observed when experimental conditions more favorable to tetramer formation are used (G. G. Johnson, unpublished data).
14. We have observed dimeric forms of the human and mouse enzymes at low ionic strength (10 m*M* potassium phosphate, *p*H 6.8) in the absence of ampholyte; therefore, it is likely that the changes in the sedimentation velocity we observed are related to ionic strength, rather than to the presence of the ampholyte or the differences in the *p*H's of the two gradients in Fig. 4.
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Chemotactic Responses of Tumor Cells to Products of Resorbing Bone

Abstract. *To explore possible mechanisms for the metastasis of malignant cells to bone, a model of tumor cell migration was developed, using Walker carcinosarcoma or malignant lymphoma cells. It was found that bone contains a factor that is strongly chemotactic for tumor cells. This factor is released by a variety of agents that induce resorption of bone.*

Bone is one of the most common sites of metastasis of tumor cells. We have developed a model for studying the migration of tumor cells toward bone, using a type of tumor cell that frequently metastasizes to bone. We found that during the process of resorption, bone releases a factor that is strongly chemotactic for the tumor cells.

The Walker carcinosarcoma is a spontaneous rat mammary tumor that causes hypercalcemia and osteolytic bone le-

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sions in vivo (*1*). Cultured Walker carcinosarcoma cells release a factor that stimulates bone resorption in vitro, a phenomenon that can be related to the propensity of tumors to metastasize to bone (*2*). Since neoplastic cells are capable of directed migration toward certain chemotactic stimuli (*3*), we looked for the production of such stimuli during the process of bone resorption. Walker carcinosarcoma cells are well suited for study in chemotaxis chambers because they grow loosely adherent to cell culture flasks and can be dislodged without trypsinization. In the experiments reported here we took culture media from fetal rodent long bones that were stimulated to undergo resorption by products of Walker carcinosarcoma cultures and tested these media for chemotactic activity for tumor cells, using modified Boyden chemotaxis chambers.

Walker carcinosarcoma 256 cells (Flow Laboratories) were maintained by serial passage in cell culture. After the cells were mechanically removed from the culture flasks, their chemotactic responses were examined in modified Boyden chambers, using nitrocellulose filter membranes with pores 12 μm in diameter (Schleicher and Schuell). A sample of 5×10^5 cells per milliliter of culture medium was placed in the upper compartment of each chamber. A 1-ml sample of the test medium to be assessed for chemotactic activity was placed in the lower compartment. After incubation for 4 hours at 37°C in 5 percent CO_2 and air, the filters were removed, fixed, and stained with hematoxylin. Cells that migrated into the filter to a depth of 15 μm were counted by light microscopy. Experiments were performed in triplicate, and five high-power fields (×400) were counted for each membrane. The mean number of cells that had migrated into the filters was determined from the resultant 15 counts and expressed as cells per high-power field. Neutrophilic leukocytes were obtained from peritoneal exudates induced in rabbits by injection of glycogen (*4*). The chemotactic responses of these cells were assayed as described previously (*5*). Data were analyzed statistically by Student's *t*-test. In some experiments the purified fifth component of complement (C5) was trypsinized and used as a known chemotactic factor for tumor cells (*6*). Antiserum to C5 was also incubated at 37°C for 30 minutes with some bone culture media and with trypsinized C5 preparations and assessed for suppression of chemotactic activity.

Chemotactic activity was assayed in the media bathing organ cultures of bone undergoing resorption. The organ culture of rodent long bones has been described in detail and is used for bone resorption bioassay (*7*). Pregnant rats on the 18th day of gestation or pregnant mice on the 16th day of gestation were injected subcutaneously with 0.2 or 0.05 mCi of ^{45}Ca, respectively. After 24 hours the mother was killed, the uterus was sectioned, and the tubular mineralized shafts on the fetal rodent radius, ulna, and fibula were dissected free of the fetal subcutaneous tissue and the muscle and cartilaginous ends were removed. The bones were cultured in control medium for 24 hours so that loosely complexed ^{45}Ca could be exchanged with the stable calcium in the medium. The bones were then cultured for 5 days with a mediator of osteoclastic bone resorption or with control media. In most of these experiments, bones were cultured with medium from cultured Walker carcinosarcoma cells. In some experiments, the bones were cultured with parathyroid hormone (400 ng/ml) or prostaglandin E_1 (10^{-7} or $10^{-5}M$). In each treatment group 12 to 24 bones were cultured. Bone resorption was assessed by measuring the percentage of the total radioactivity released into the medium from individual bones during the period of culture. Data were analyzed statistically by Student's *t*-test.

Table 1 shows the relative migration of Walker carcinosarcoma cells and leukocytes moving from the upper compartments of the Boyden chambers toward the lower compartments, which contained a variety of potential chemoattractants. As indicated by ^{45}Ca release, bone resorption was stimulated when bones were cultured with the supernatants from cultured Walker carcino-

Table 1. Chemotactic responses of Walker carcinosarcoma cells and rabbit peritoneal leukocytes to products of resorbed rodent long bones. Experiments are referred to as (A) and (B).

Chemoattractant	Bone resorption (percentage of ^{45}Ca release)*		Chemotactic activity (cells migrated per HPF)†			
			Tumor cells		Leukocytes (*14*)	
	(A)	(B)	(A)	(B)	(A)	(B)
Media from bones incubated with supernatant fluids from tumor cell cultures	37 ± 3‡	74 ± 2‡	45 ± 4‡	45 ± 3‡	17 ± 5	12 ± 2
Media from untreated bones	28 ± 2	47 ± 2	20 ± 2	26 ± 1	27 ± 2‡	16 ± 2
Supernatant fluids from tumor cell cultures			21 ± 1	17 ± 1	15 ± 3	18 ± 2
Control medium			29 ± 2	21 ± 2	12 ± 2	26 ± 4
Leukocyte chemotactic factor from C5 (75 μl/ml)§			21 ± 2	17 ± 1	74 ± 6‡	58 ± 6‡
Tumor cell chemotactic factor from C5 (75 μl/ml)‖			35 ± 2‡	43 ± 2‡	25 ± 2‡	34 ± 2
Escherichia coli culture fluid			20 ± 1	21 ± 1	59 ± 7‡	70 ± 4‡

*Each value is the mean ± standard error (S.E.) for four bone cultures. †Each value is the mean ± S.E. for 15 four high-power fields (HPF) (tumor cells) or 10 HPF (leukocytes). ‡Significantly different from corresponding control ($P < .05$). §Leukotactic fractions of activated serum obtained by chromatography on Sephadex G100. ‖Leukotactic fractions of activated serum (as above) digested with 1 percent trypsin (by weight) for 30 minutes at 37°C. Digestion was stopped with 2 percent (by weight) soybean trypsin inhibitor.

Table 2. Effects of different humoral mediators of bone resorption on the generation of chemotactic activity from resorbing bones.

Mediator of bone resorption	Bone resorption (percentage of ^{45}Ca release)	Chemotactic activity (cells migrated per HPF)*	
		Walker cells	EL-4 cells
Experiment 1			
Supernatant fluids from tumor cell cultures			
Bone culture fluid undiluted	74 ± 4†	43 ± 2†	4 ± 0
Bone culture fluid diluted 1:8		18 ± 1	16 ± 1†
Control medium	25 ± 1	19 ± 1	5 ± 0
Experiment 2			
Prostaglandin E_1 ($10^{-5}M$)	47 ± 2†	43 ± 2†	10 ± 1
Prostaglandin E_1 ($10^{-7}M$)	44 ± 2†	31 ± 2†	32 ± 2†
Control medium	25 ± 1	24 ± 1	16 ± 2
Experiment 3			
Parathyroid hormone (400 ng/ml)	40 ± 3†	18 ± 2†	
Control medium	21 ± 2	5 ± 1	

*Each value is the mean ± S.E. for 15 HPF. †Significantly different from corresponding control ($P < .05$).

sarcoma cells. Media from bone cultures in which resorption had occurred attracted significantly more tumor cells into the membrane filters than media from the untreated bones. Chemotactic activity was not detected in the supernatant fluids from cultured Walker carcinosarcoma cells, in the supernatant fluids from devitalized bones (data not shown), or in the control media. The tumor cells also migrated to a digestion product of the C5 leukotactic peptide isolated from activated serum (*6*), but not to the bacterial factor generated in cultures of *Escherichia coli*. In contrast, rabbit neutrophils did not migrate to the media from resorbed bone but were attracted by the bacterial factor and the C5 peptide isolated from activated serum.

Migration of tumor cells to resorbed bone media was independent of the humoral mediator of bone resorption (Table 2). Bones that were stimulated to resorb by supernatant fluids from cultured tumor cells, parathyroid hormone, or prostaglandin E_1 all released factors that stimulated the movement of tumor cells into the membrane filters. The generation of chemotactic activity was dependent on resorption of the bones. A second tumor line, the EL-4 murine lymphoma (*8*), was also attracted by media from resorbing bones. Like the Walker carcinosarcoma cells, the cells of this tumor responded to bone-derived factors regardless of the mediator of bone resorption. However, the responses of these cells were observed most readily when the bone culture fluid was diluted. Unlike the Walker carcinosarcoma cells, the EL-4 cells did not respond to the C5-derived chemotactic factor for tumor cells (*6*).

The responses of the tumor cells could not be ascribed solely to chemokinesis (increased random cell migration). This was shown by adding medium from resorbed bones to the upper compartments of the Boyden chambers or to both the upper and lower compartments. Maximum migration occurred when the stimulus was in the lower compartment on the side of the membrane opposite the cells (Table 3).

The specificity of the chemotaxis of cultured tumor cells to resorbed bone media is unknown at present. The bone-derived factor is not chemotactic for neutrophils (Table 1) or lymphocytes (*9*). We found that circulating human monocytes were attracted to the products of resorbing bones, and this may explain the frequent appearance of monocytes at the margins of resorbing bones (*9*). Since tumors of most histological types will metastasize to bone (*10*), chemotactic responses to bone-derived products may be a property of many different neoplastic cells. It will be necessary to survey a large number of tumor cell lines to determine how frequently cultured tumor cells respond to the bone-derived factor and whether this response can be correlated with the propensity of the tumor cells to metastasize to bone in vivo. In preliminary studies we found that cultured mouse myeloma cells that form localized intraperitoneal tumors in vivo do not respond to the bone-derived chemotactic factor in vitro.

Table 3. Demonstration that the migration of tumor cells is chemotactic in nature.

Upper compartment	Cells migrated per HPF* when lower compartment contains	
	Control media	Resorbed bone media†
Control media	21 ± 1	35 ± 4
Resorbed bone media	23 ± 2	17 ± 3

*Each value is the mean ± S.E. in 15 HPF. †Mean release of ^{45}Ca from bones is equal to 55 ± 4 percent of total.

Our findings suggest that bone contains a factor that is chemotactic for tumor cells and is released when bone is resorbed. Since normal bone is constantly being remodeled by the coupled processes of resorption and formation, factors chemotactic for tumor cells could be released from endosteal bone surfaces continuously. The release of chemotactic factors from bone might be initiated or greatly amplified by tumor cells that have migrated randomly to the vascular bone marrow. These tumor cells could produce local factors such as prostaglandins (*2*) or other factors that stimulate adjacent osteoclastic bone resorption, leading to release of the bone-derived chemotactic factor.

The chemical nature of the bone-derived chemotactic factor for neoplastic cells is unknown at present, but preliminary experiments show that it is stable when heated at 56°C for 60 minutes. It is retained by dialysis membranes with a molecular weight cutoff of 3500 and is lost when the cutoff is 10,000. Incubation of culture media from resorbing bones with antiserums to C5 had no effect on the chemotactic activity, but this treatment completely blocked the chemotactic activity for tumor cells generated by trypsinization of C5. Thus the two chemotactic activities appear to be derived from different sources. The chemotactic factor for tumor cells may be a fragment of the proteinaceous bone matrix released during the process of bone resorption. Enzymatic products of other types of collagen have been shown to be chemotactic for fibroblasts (*11*).

Although it has been known for several years that certain tumor cells respond to chemotactic stimuli both in vitro and in vivo, there have been very few studies of tumor cell chemotactic factors. Ushijima *et al.* (*12*) described the characteristics of several proteins extracted from tumor tissue that are chemotactic for tumor cells in vitro and in vivo. We showed that C5 is the source of a tumor cell chemotactic factor. This complement-derived tumor cell chemotactic factor is distinct from the complement leukotactic factor but is probably derived from it (*6*). The pathophysiologic significance of these factors is not clear at present. It is possible that the C5 could be cleaved by proteolytic enzymes produced by the tumor cells or even normal cells to form a chemotactic factor.

Human tumors often metastasize to bone. Breast cancer, the most common malignant disease of women in the United States, metastasizes to bone more frequently than to any other organ, and approximately 70 percent of patients who die with breast cancer have bone metastases (*13*). Bone lesions cause considerable morbidity in terms of pain, pathological fractures, and hypercalcemia, and once tumor metastases are found in bone the disease is incurable. We believe that our observations of a bone-derived chemotactic factor for tumor cells may provide a means for studying the pathophysiology of cancer cell metastasis to bone in vitro. This may be of use in the development of rational therapeutic programs to prevent or inhibit this process.

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Tumor Surveillance: How Tumors May Resist Macrophage-Mediated Host Defense

Abstract. *Both normal human serum and supernatant from explanted malignant tumors contained a heat-stable low-molecular-weight factor that inhibited monocyte activation in vitro. In contrast, serum from individuals with solid tumors enhanced monocyte activation. It is suggested that the systemic activation of monocytes that occurs in malignant disease may be an appropriate host response but that successful tumors may continue to grow because they subvert the normal physiological signal for inhibition of macrophage activation.*

A considerable body of evidence shows that cells of the mononuclear phagocyte series can recognize and kill transformed (*1*) and malignant (*2*) cells, and a defensive role in host surveillance against neoplastic disease has been ascribed to the macrophage (*3*). Investigations of monocyte or macrophage function in tumor-bearing hosts have shown that the capacity to mobilize macrophages into inflammatory sites may be defective (*4*) and that the chemotactic responses of monocytes may be depressed (*5*). Moreover, studies in both animal (*6*) and human (*7*) systems indicate that this depression of monocyte function is mediated by tumor products. Snyderman and his colleagues have argued that by this mechanism tumors may escape macrophage-mediated surveillance (*8*).

Monocytes and macrophages possess membrane receptors for the Fc portion of immunoglobulin G (Fc receptors) (*9*), the functional expression of which is markedly increased during cellular activation in vivo (*10, 11*) and in vitro (*11*). Changes in the expression of this receptor can be conveniently assayed to provide a reliable indicator of cellular activation or depression (*11–13*). The peripheral blood monocytes of individuals with solid malignant tumors (*14*) and lymphomas (*15*) exhibit an increased expression of Fc receptors and this change does not occur readily in a variety of nonmalignant diseases (*14*). Experiments in guinea pigs suggest that macrophage activation is controlled by an inhibitory factor in normal serum (*11*). We therefore wished to look for factors in human serum and tumor supernatants that might be modulating macrophage function. Here we show that the activation of normal human monocytes in vitro is inhibited by a factor in normal human serum but enhanced by serum from individuals with solid malignant tumors. We show that tumors release a factor resembling that present in normal serum, which inhibits monocyte activation in the same manner.

Fresh serum samples were obtained from untreated patients with primary carcinomas of the lung or colon. The majority were free of obvious infections and metastatic disease. Control serums were obtained from normal donors of comparable age. No age- or sex-related differences in monocyte function or in serum effects on monocyte function were observed. Studies of soluble tumor products in vitro were performed largely on freshly excised superficial bladder carcinomas because of the discrete nature of the tumor mass, its sterility, and the dissociability of the cells, and because normal mucosa from an adjacent area could be easily identified and sampled by transurethral resection. Squamous cell carcinomas of the lung and adenocarcinomas of the breast were also studied.

Normal mononuclear cells were isolated from defibrinated blood by centrifugation over a Ficoll-Triosil gradient. The cells were washed twice in Hanks balanced salt solution (BSS) and resuspended at a concentration of 2×10^6 per milliliter in RPMI 1640 containing 20 percent heat-inactivated fetal calf serum (FCS). A 1-ml portion was placed in each chamber of dual tissue culture slides (Lab-Tek Products). After incubation at 37°C for 1 hour, the nonadherent cells were removed by washing the monolayer with serum-free medium. The presence of 20 percent serum during monocyte adherence is important because it prevents the adherence of lymphocytes. Platelets were removed in the defibrination procedure. Approximately 95 percent of the adherent cells at this stage are monocytes. After 24 hours of culture, contaminating lymphocytes and granulocytes, identified by Giemsa staining, were reduced to less than 2 percent.

Samples of tumor tissue and normal bladder mucosa were washed and gently minced with scissors in cold RPMI 1640 containing antibiotics. The suspension was adjusted to 10 percent by volume and cultured for 24 hours at 37°C, after which cell-free supernatants were obtained by centrifugation. In some experiments a single cell suspension was obtained by pressing the tissue through a fine steel mesh. Adherent cells were then depleted by allowing the cells to settle onto glass for 90 minutes, after which nonadherent cells were collected by aspiration. Ultrafiltration of supernatants was performed by using cone filters with more than 95 percent retention for molecules larger than 50,000 or 25,000 daltons (Amicon Centriflo membranes, type CF50A or CF25), following the manufacturer's instructions. Ultrafiltration of fresh serum samples was performed in the same way. Heat inactivation was carried out by incubating ultrafiltrates at 56°C for 35 minutes. Normal monocytes that adhered under the uniform conditions described were then cultured in RPMI 1640 containing antibiotics supplemented with the serums or supernatant preparations at the concentrations specified. No significant differences in cell loss or viability were observed. After culture, the cells were washed in serum-free BSS and the assay for Fc receptor expression was performed in BSS in the absence of any serum. A panel of ox erythrocytes bearing increasing amounts

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