The Chemotactic Response of Tumor Cells

A Model for Cancer Metastasis

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Injection of a C5-derived chemotactic factor for tumor cells into the peritoneal cavities of Sprague–Dawley rats induced diffuse mesenteric metastasis following the intravenous injection of Walker carcinosarcoma cells. Intraperitoneal injections of culture medium, histamine, or of trypsin-treated albumin resulted in many fewer metastases. Intraperitoneal injections of the chemotactic factor, unlike histamine, did not alter mesenteric vasopermeability as measured by the exudation of Evans blue into the mesentery. *In vitro*, tumor cells responded to the chemotactic factor by demonstrating directed migration in the Boyden chamber, by volume changes, measurable in the Coulter counter, and by demonstrating an increased adherence to nylon fibers. These phenomena are similar to the behavior of neutrophils in the presence of their chemotactic factors. All the responses *in vitro* were markedly depressed by the addition of 2-deoxyglucose, while the cell swelling response was slightly enhanced by cytochalasin B (again similar to the responses of leukocytes). The data suggest that movement of tumor cells from the circulation may be under chemotactic influence in a manner similar to the responsiveness of neutrophils to leukotactic stimuli *in vivo*. (Am J Pathol 1981, 104:69–76)

THE MIGRATION of leukocytes out of blood vessels appears to be controlled by chemotactic signals that are generated from a variety of sources, especially the complement system.[1] In acute inflammatory reactions, such as those triggered by tissue deposition of immune complexes, biologically active mediators (especially C5a from the fifth component of complement [C5]) seem to play a key role in directing the movement of neutrophils out of the bloodstream.[2] By the use of a variety of *in vitro* techniques it has been possible to demonstrate that chemotactic factors react with receptors on the surfaces of neutrophils, resulting in cationic fluxes across the cell membrane, enzyme activation, the production of oxygen metabolites, enzyme secretion, aggregation and swelling of activated cells, and, finally, cell movement (reviewed in Zigmond[3] and O'Flaherty and Ward[4]). Some of these events, such as cell swelling and aggregation, can be monitored *in vitro* in a rapid and sensitive manner.[5]

Malignant cells respond *in vitro* to chemotactic stimuli that are structurally different from the chemotactic factors of leukocytes. These chemotactic factors derive from tumor tissue,[6] resorbing bone,[7] and C5.[8] The C5-related factor can be generated with the use of a variety of neutral proteases, with intact C5 or C5a as a substrate.[9-10] When the latter is reacted with trypsin, it is converted into a factor (mol wt $\sim$ 6000) that is devoid of leukotactic activity but demonstrates distinct chemotactic activity for tumor cells.[9,11] We have shown that neutral proteases (such as elastase), released either from inflammatory cells or from normal tissues, will act on C5, resulting in the production of a peptide that is chemotactic for tumor cells.[10,12] The production of this signal *in vivo* could presumably cause the efflux of tumor cells from the bloodstream into the area in which the peptide has been generated.

In the work reported here we provide further evidence that, *in vitro* and *in vivo*, the responses of tumor cells to chemotactic agents are similar to the responses

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of leukocytes to leukotactic factors. Just as in the case of neutrophils, tumor cells exhibited directed migration, volume changes, and increased adherence to nylon fibers following appropriate stimulation by chemotactic factor for tumor cells. *In vivo*, the same chemotactic agent induced diffuse local metastasis when injected into animals with circulating tumor cells. The data affirm the biologic activity of chemotactic factors for tumor cells and suggest that movement of tumor cells from the circulation may be under chemotactic influence in a manner similar to the *in vivo* responsiveness of neutrophils to leukotactic stimuli.

Materials and Methods

Cells

The tumor cells used in this study were Walker carcinosarcoma cells maintained in the ascities form by serial intraperitoneal passage of tumor cells in Sprague–Dawley rats. The preparation of these cells for experimental studies was described in detail previously.[9]

Chemotactic Factors

The chemotactic factor for tumor cells was derived from C5a-containing fractions obtained by gel filtration of zymosan-activated human serum. The C5a-containing material was treated with trypsin (1% protein wt/wt) to generate the chemotactic factor. These procedures are fully described in a recent publication.[9]

Assay of Cell Migration and Chemotaxis

The standard Boyden chamber assay for tumor cell chemotaxis was performed as described in several of our recent publications.[9,10] Twelve-micron filters (Schleicher and Schuell Company, Keene, New Hampshire) were used in this study. Each assay was performed in triplicate, and the number of cells migrating in three high-power fields was determined on each filter. The mean and standard error were calculated from these values. Statistical analysis was performed with the use of the Student *t*-test.

Particle Counter Assay

A suspension of tumor cells was prepared in Hanks' balanced salt solution at a concentration of 1×10^4 cells/ml and counted with a Coulter Counter, Model ZBI. The chemotactic factor was added to the cell suspension, and cell counts were made 30 seconds later and at 1-minute intervals over a 10-minute period. Using a Coulter Channelyzer C-1000, the mean cell volume distribution was measured at the same time intervals. This assay procedure is similar to that described by O'Flaherty et al.[5]

Nylon Fiber Adherence

The assay described by MacGregor et al[13] was modified to measure tumor cell adherence. We prepared a nylon-fiber column by packing 10-ml syringes with 100 mg of scrubbed nylon wool (Associated Biomedic Systems, Buffalo, NY). The syringes, equipped with two-way stopcocks, were packed from the bottom to the 3-ml mark. One ml of Hanks' balanced salt solution containing 5×10^6 cells was treated with 100 μl of solution containing the chemotactic factor or an appropriate control substance and was added to the column. After allowing the cells to adhere for 3 minutes, the stopcocks were opened and nonadherent cells were washed through with 20 ml of phosphate-buffered saline. The number of cells washed out of each column was counted, and the percentage of adhering cells was determined. In each experiment the assay was performed in triplicate.

Assay of Tumor Metastasis

To study the accumulation of tumor cells *in vivo*, 2×10^7 Walker cells were injected intravenously into the tail veins of female Sprague–Dawley rats weighing 100–150 g. At the time of injection of tumor cells and daily for each of the next 4 days, 500 ED_{50}'s (approximately 500 ng C5-related peptide) of tumor cell chemotactic factor was injected into the peritoneal cavity of each rat. Controls included animals given intravenous injections of tumor cells followed by the intraperitoneal injections of Hanks' balanced salt solution or trypsinized albumin. The total number of grossly visible metastatic tumors along the mesentery of the small intestine was counted on the seventh day.

Vasopermeability Assay

One milliliter of a 1% aqueous solution of Evans blue, in Hanks' balanced salt solution, was injected into the tail veins of female Sprague–Dawley rats (100–150 g). Immediately after injection of the dye, one group of animals received intraperitoneal injections of 500 ED_{50} of trypsinized C5a. Controls included animals injected intraperitoneally with Hanks' balanced salt solution, trypsinized serum albumin, or the vasoactive agent, histamine. One hour later, the mesenteries were excised and lyophilized for 48 hours to remove all water. The dye in the mesentery was ex-

tracted by the modified method of Taichmann and Movat.[14] A random sample of lyophilized mesentery was weighed and placed into 3 ml of formamide. The dye was extracted for 3 days at 45 C. Optical density of the extracted dye was measured at 620 nm in a spectrophotometer. Using a calibrated curve, the amount of extracted dye was calculated in terms of micrograms of Evans blue per milligram of dry tissue.

Results

Effects of the C5-Derived Chemotactic Factor *in Vitro*

The Boyden chamber assay for directed migration,[9] the particle counter assay for cell swelling and cell-to-cell aggregation,[5] and the nylon fiber assay for cell adherence[13] were used. These assays were chosen because previous work with leukocytes had shown that all three responses occurred upon interaction of the cells with chemotactic factors[15] and that these *in vitro* responses correlated with *in vivo* responses to the same factors.[16]

In the Boyden chamber assay the C5a-derived tumor cell chemotactic peptide induced a dose-dependent chemotactic response similar to that described previously.[9,11] Neither Hanks' balanced salt solution, trypsinized serum albumin, nor histamine had this effect (Figure 1). As demonstrated in previous publications,[8,9] C5a-containing fractions of activated serum produced no effect on the migration of tumor cells in the Boyden chamber (data not shown).

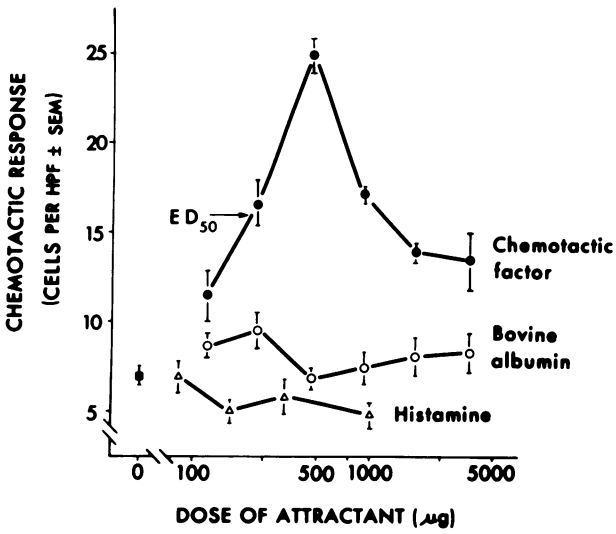

Figure 1—Chemotactic response of Walker ascites tumor cells to various doses of several agents. The chemotactic factor (● ——●) was the trypsin-treated C5a-containing fraction of zymosan-activated serum, prepared according to details in the text. Bovine serum albumin (O——O) at the same protein concentration as the chemotactic factor was digested with trypsin in an identical manner. Histamine (△——△) was dissolved in Hanks' medium (■). The dose of attractant represents micrograms of each agent suspended in a volume of 1 ml in Hanks' balanced salt solution.

Table 1 compares the behavior of the tumor cells in the Boyden chamber, the particle counter assay, and the adherence assay. In the particle counter assay, the C5a-derived tumor cell chemotactic factor induced a drop in the cell count of approximately 30% as measured with the Coulter Counter, Model ZBI. The drop in the cell count was rapid (the maximum response occurring in 1–3 minutes) and was reversible (cell numbers returned to baseline in 5–7 minutes). Using a volume analyzer, we found the drop in particle count to be accompanied by an increase in cell diameter of approximately 20%. These findings with regard to the effects of the chemotactic factor on cell swelling are in accord with similar effects of C5a on neutrophils.[5] However, in contrast to these studies on leukocytes, we did not observe discrete second and third peaks on the mean cell volume plot that would correspond to 2-and 3-cell aggregates. Adherence of the tumor cells to nylon fibers was increased by treatment of the cells with the chemotactic factor for tumor cells. The precursor, C5a-containing fractions of activated serum, produced a marginal effect in this assay.

Additional experiments (not shown) demonstrated that treatment of the tumor cells with 10^{-3} M 2-deoxyglucose completely blocked the responses of the cells to the chemotactic stimuli in the Boyden chamber assay, in the particle counter assay, and in the adherence assay. Furthermore, treatment of the tumor cells with 0.5 μg/ml cytochalasin B potentiated their response to the chemotactic factor as measured in the particle counter assay but not in the Boyden chamber assay. The influences of these drugs on the chemotactic response of leukocytes are remarkably similar.[17]

Effects of the C5-Derived Chemotactic Factor *in Vivo*

The mesentery was chosen as the organ for this study because of its structural similarities to the Boyden chamber. The entire mesentery is suspended freely in the peritoneal cavity and is thus allowed to come into contact with peritoneal fluid. The mesenteric vessels are easily seen between two layers of transparent membrane. The intravenous tumor cells have to transverse the vascular endothelium to reach the interstitium, where they proliferate to form observable metastatic nodules (Figure 2). The metastatic tumors appeared grossly as discrete, white nodules along the plane of the mesentery. They were easily counted when the mesentery was spread out. Histologically,

Table 1—*In Vitro* Responses of Tumor Cells to the C5-Derived Chemotactic Factor

	Chemotactic Response* (Cells/HPF ± SEM)		Particle Count† (Percent decrease ± SEM)		Adherence‡ (Percent of total ± SEM	
Chemoattractant	Exp A	Exp B	Exp A	Exp B	Exp A	Exp B
Hanks' balanced salt solution	0	0	2 ± 1	1 ± 0	24 ± 3	20 ± 5
Bovine serum albumin	0	0	2 ± 1	2 ± 1	—	—
C5a-containing precursor	0	2 ± 1	2 ± 1	2 ± 1	31 ± 6	28 ± 4
Chemotactic factor for tumor cells	18 ± 3§	20 ± 4§	29 ± 4§	31 ± 5§	43 ± 5§	42 ± 8§

* Boyden chamber assay. Values represent the number of cells that have migrated per HPF above values obtained when Hanks' balanced salt solution was used as a chemoattractant (random migration).

† The various chemoattractants were added to suspensions of cells. Aliquots of each suspension were immediately assayed in the ZBI Coulter Counter over a period from 30 seconds to 10 minutes. The values shown in the table represent the maximum percent decrease in cell counts. Each value is the mean of 5 separate experiments; in each experiment the samples were assayed in triplicate.

‡ Percentage of cells that were retained in the nylon fiber column after an incubation period of 3 minutes and elution of the column with 20 ml of phosphate-buffered saline. The values obtained in one experiment are shown.

§ $P \leq 0.05$ when compared with the corresponding control.

the tumor nodules were located within the mesenteric tissue, around blood vessels, rather than on the mesothelial surface. Figure 3 illustrates the gross and histologic appearance of the mesenteric metastases.

As shown in Figure 4, in a total of 84 animals (from experiments with 3 groups per experiment), those rats given an intraperitoneal injection of the tumor cell chemotactic factor showed a 2.5–7-fold increase in the number of metastatic nodules developing in the mesentery, as compared with the injection of trypsinized albumin, which caused a much smaller increase in the numbers of metastatic nodules, and the injection of Hanks' solution, which caused very few metastatic nodules to form. We were unable to demonstrate that intraperitoneal injection of the various agents altered the pattern of tumor metastasis to organs other than the mesentery.

Since C5a is likely to contribute to the increased vasopermeability seen in the acute inflammatory reaction,[18] we wondered whether the chemotactic factor derived from C5a might act to cause enhanced vascular permeability. The widening of the endothelial gap

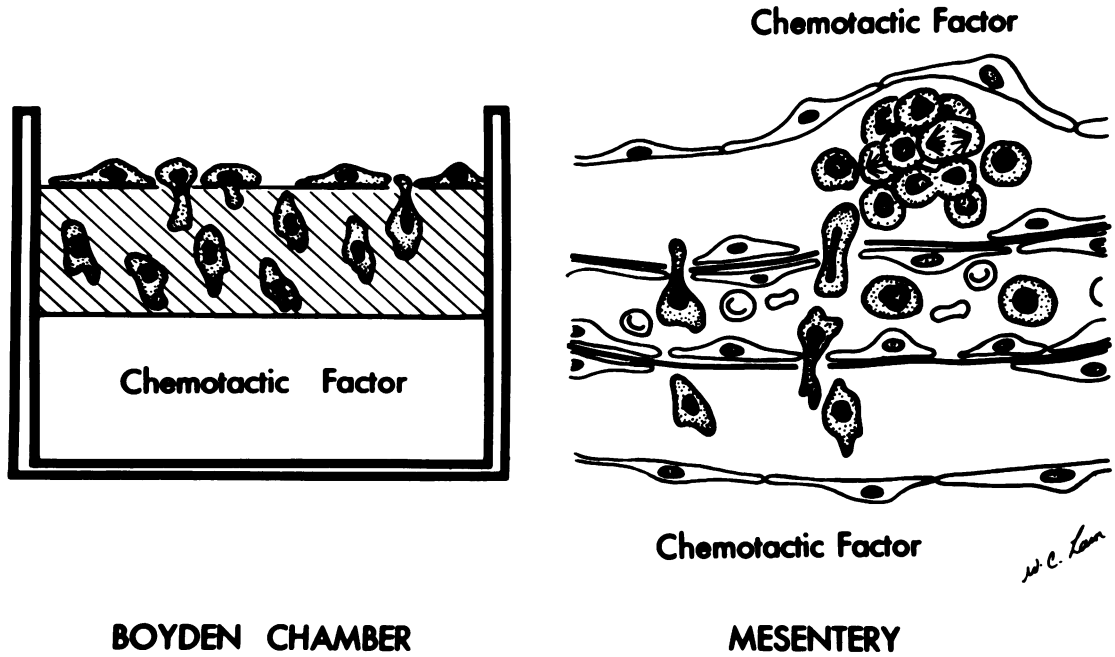

Figure 2—Similarities between the Boyden chamber, used to measure chemotaxis *in vitro*, and the rat mesentery, used to measure metastasis *in vivo*. In the Boyden chamber, cells migrate from the upper chamber into the interstices of a nitrocellulose filter, which separates the cells from the lower compartment of the chamber, into which the chemoattractant has been placed. The relative depth of the filter has been exaggerated in this diagram in order to indicate that, in our assay, the cells counted are those that have migrated to a depth of ~ 25 μ after 4 hours' incubation. (See Orr et al[9]). The mesentery is bathed by peritoneal fluid, into which the chemotactic factor is placed for experiments *in vivo*. Circulating tumor cells enter the extravascular tissues, where they proliferate to form grossly visible metastases.

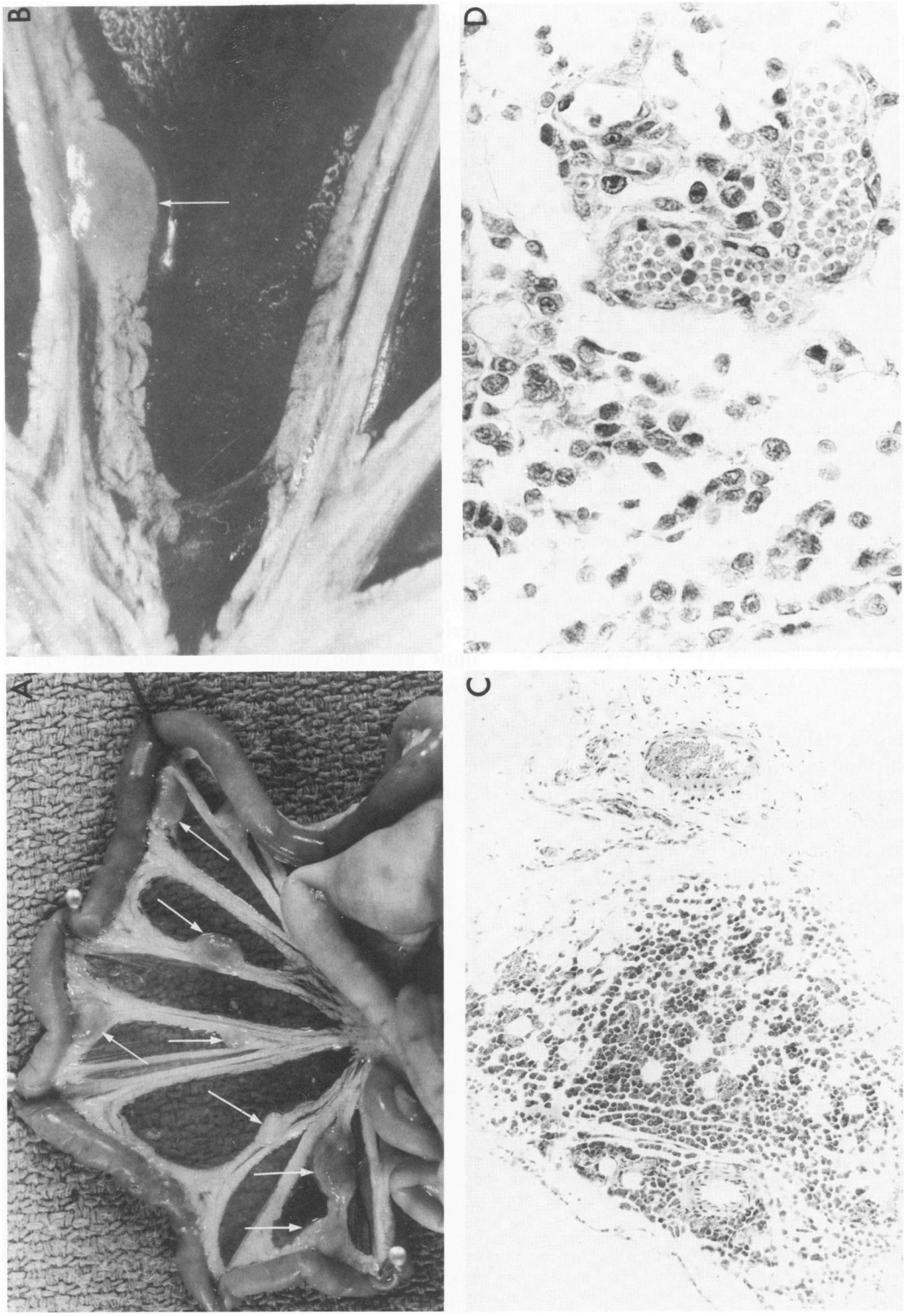

Figure 3—Gross and histologic appearance of the mesenteric metastases of Walker carcinosarcoma cells induced by intraperitoneal injections of chemotactic factors for tumor cells. **A**—View of a segment of the small bowel mesentery. The *arrows* point to several of the metastatic nodules. **B**—Metastases are located along the mesenteric vascular arcades. **C**—The tumors are located within the mesenteric tissue rather than on the mesothelial surface. (H&E, ×250) **D**—The anaplastic tumor cells are located in relationship to small and large blood vessels within the mesentery. (H&E, ×400)

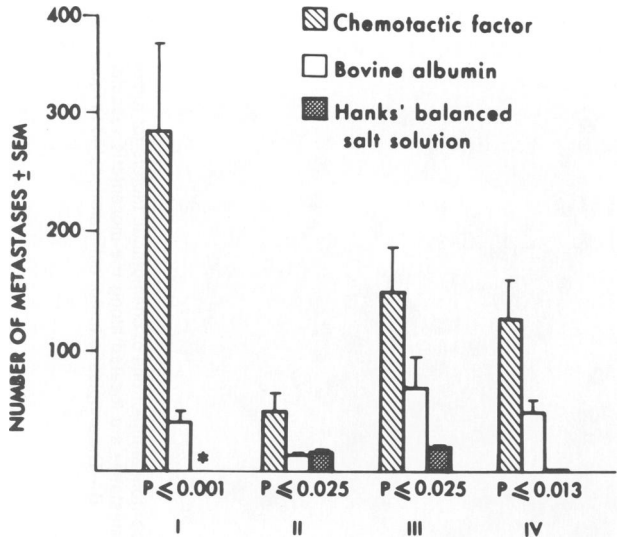

Figure 4—Induction of metastases in the rat mesentery by intraperitoneal injection of a chemotactic factor for tumor cells. The results of 4 separate experiments are shown. The chemotactic factor was prepared by trypsinizing partially purified C5a, obtained from zymosan-activated serum. (See text and Orr et al[9] for details). Five hundred ED_{50} of the factor in Hanks' balanced salt solution were injected daily for 4 days into the peritoneal cavities of female Sprague-Dawley rats. Bovine serum albumin, at the same protein concentration as the chemotactic factor, and Hanks' balanced salt solution were trypsinized in a manner similar to the chemotactic factor. Identical volumes of these two agents were then injected daily for 4 days into the peritoneal cavities of control animals. On the 1st day 2×10^7 Walker carcinosarcoma ascites cells were injected intravenously. On the seventh day grossly visible metastases were counted along the mesenteric vessels.
* Not done.

could allow passive efflux of circulating tumor cells into the interstitium, accounting for the increased number of metastatic nodules in the experimental group. We therefore examined the effect of the chemotactic factor for tumor cells on the vasopermeability of the mesentery. The results are shown in Table 2. The amount of dye extracted from the mesentery of the trypsinized C5a-treated animals was the same as that extracted from animals treated with Hanks' solution or bovine serum albumin. On the other hand, in animals treated with histamine, a known vasopermeability agent, about twice the amount of dye was extracted from the mesenteries. These observations indicate that the trypsinized C5a does not appear to cause enhanced vasopermeability and suggest that the increase in mesenteric nodules seen after injection of trypsinized C5a is not due to enhanced vasopermeability. We then decided to determine whether the vasopermeability agent histamine would cause increased localization of tumor cells. According to the previous method, 2.0×10^7 cells were injected into the rats via the tail vein. At the time of injection of cells and daily for the next 2 days, 1000 μg of histamine, dissolved in 15 ml of Hanks' solution, was injected into the peritoneal cavity of the rat. Similarly, the same volume of Hank's solution and bovine serum albumin (at the same protein concentration, and trypsinized in the same way, as the chemotactic factor) were injected into the peritoneal cavities of the control animals. On the 7th day after the injection of cells, the metastatic nodules were counted along the mesenteric vessels. As shown in Table 2, in 2 experiments, no statistically significant difference was found in the number of mesenteric nodules between the group treated with histamine and the control groups treated with either Hanks' solution or bovine serum albumin. This result supports the previous observation of Ozaki et al[18] that enhanced vasopermeability alone will not cause significant extravascular emigration of circulating tumor cells.

Discussion

The present study, taken together with previous reports, suggests that there is a remarkable parallelism

Table 2—Effect of Intraperitoneal Injection of Various Agents on Mesenteric Vasopermeability and the Development of Mesenteric Metastasis

Intraperitoneal injection	Vasopermeability effect* μg Evans blue/mg dry tissue ± SEM		Mesenteric metastases† (Number of nodules/rat ± SEM)		
	Exp A	Exp B	Exp C	Exp D	Exp E
Hanks' balanced salt solution	212 ± 42	220 ± 49	7 ± 4	15 ± 1	—
Histamine (1000 μg)	535 ± 53¶	438 ± 25¶	16 ± 14	—	4 ± 2
Chemotactic factor (500 ED_{50})‡	—	245 ± 30	—	50 ± 17¶	27 ± 11¶
Bovine serum albumin§	—	267 ± 16	16 ± 5	14 ± 2	4 ± 3

* One milliliter of 1% Evans Blue in Hanks' solution was injected intravenously. The test factor in a volume of 15 ml of Hanks' solution was then injected into the peritoneal cavity. One hour later, the mesenteries were excised and lyophilized. Dye was extracted from samples of lyophilized mesentery into 3 ml of Formamide for 3 days at 45 C. The absorbance was measured at OD_{620}.
† Walker carcinosarcoma cells, 2.0×10^7, were injected intravenously. Test factor was injected daily for 4 days into the peritoneal cavity. The number of mesenteric metastases was counted on the seventh day.
‡ Chemotactic factor was generated by trypsinization of C5a-containing fractions of zymosan-activated serum as described in the text.
§ Treated in a manner identical to the chemotactic factor (see text).
¶ $P \leq 0.05$ when compared with corresponding control.

between the response of tumor cells and leukocytes to chemotactic factors. *In vitro*, both cell types show chemotactic migration, cell volume expansion, and hyperadherence in response to chemotactic factors. It is demonstrated here, furthermore, that in tumor cells, as in leukocytes, the responses to the chemotactic factor *in vitro* correlate with responses to the same factors *in vivo*. These analogous responses indicate that there are quite likely to be underlying similarities in the mechanisms of the responses of the different cell types to their respective chemotactic factors. For instance, it can be predicted that the large body of information concerning leukocyte-chemotactic factor interaction may be applicable to further study of the chemotaxis of tumor cells where considerably less is known. The particle counter assay may be a useful rapid method of assaying tumor cell chemotactic factor activity, since this assay is much less time-consuming than the Boyden chamber assay.

This paper presents the first direct evidence that the derivative of C5a, which is chemotactic for tumor cells *in vitro*, has the ability to bring about the accumulation of chemotactically responsive cells *in vivo*. These results support the earlier observation of Ozaki et al, who, working with hepatoma cells, described the ability of a chemotactic factor derived from tumor tissue to induced intradermal localization of circulating tumor cells.[18]

The exact mechanism through which the *in vivo* accumulation of circulating tumor cells is induced by chemotactic factor is not known. One hypothesis that would take into account the observations to date is that the cell swelling response and increased adherence of tumor cells, initiated by exposure to chemotactic factor, contribute to the mechanism whereby circulating tumor cells become sequestered at secondary sites *in vivo*. After the initial arrest of the cells, they could migrate through the endothelial junction to the extravascular locale where the chemotactic signal has originated. Our data indicate that these steps are unlikely to be due alone to the action of vasopermeability agents, although it is difficult to imagine that widening the endothelial junction would strengthen the barrier between the intravascular circulating cells and the extravascular tissues.

There is evidence that some tumors have a propensity to metastasize to sites of inflammation,[19,20] and our previous studies *in vitro* have shown that conditions necessary for the generation of the C5-related chemotactic factor could occur at such locations.[10] It may be that the slight enhancement of metastasis induced by the intraperitoneal injection of bovine serum albumin is the reflection of an inflammatory response to this foreign protein.

There are several points of resemblance between the processes of cancer metastasis and inflammation. In both, cells circulate in the vasculature, stop, and cross vessel walls, thereby entering the extravascular tissues.[21,22] In the case of neoplastic cells, their subsequent proliferation results in the formation of grossly visible metastatic tumors. In this paper we have emphasized that tumor cells share with leukocytes functional characteristics that could contribute to their ability to leave the circulation in response to extravascular signals. These studies emphasize the possible broad biologic significance of tumor cell chemotactic responses in the process of metastasis.

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