An Inhibitor of Macrophage Chemotaxis Produced by Neoplasms

Abstract. *The accumulation of macrophages at neoplastic sites may be an important event in immunologically mediated tumor killing. The implantation of syngeneic neoplasms in mice, however, was found to depress the animal's ability to localize macrophages at inflammatory sites. A low-molecular-weight (6,000 to 10,000) factor released by growing neoplasms that inhibits the accumulation of macrophages in vivo and chemotactic responsiveness in vitro was identified. The factor is active in the inhibition of macrophages and is ineffectual at retarding the migration of polymorphonuclear leukocytes. Neoplastic cells may thus abrogate immunosurveillance by releasing products that prevent potentially tumoricidal macrophages from accumulating at sites of developing malignancies.*

The role of immunosurveillance in preventing the spontaneous development and spread of neoplasms remains unclear; however, it has been demonstrated that cellular immune inflammatory responses occurring within tumors can produce their destruction (*1*). Immunologically mediated tumor killing is frequently associated with the influx of macrophages to the neoplastic site (*2*). It thus seemed reasonable to hypothesize that if surveillance against tumor development or spread depended in part on macrophage localization at sites of neoplastic transformation, then hosts with depressed macrophage chemotactic responsiveness would be less likely to destroy developing tumors. We and others have demonstrated that the monocyte chemotactic responsiveness in vitro of a sizable number of humans with neoplastic diseases was depressed (*3*) and that in patients with depressed chemotaxis, surgical removal of the tumors resulted in a rapid enhancement of monocyte function (*4*). This latter finding suggested that neoplasms themselves could depress monocyte chemotaxis and perhaps thereby suppress the host's ability to retard the tumor's growth. This contention was supported by the demonstration that implantation of syngeneic neoplasms in mice produced a rapid and dramatic depression in the animal's ability to mobilize macrophages to sites of delayed inflammatory reactions in vivo (*5*).

We now report that depressed macrophage mobilization in tumor-bearing mice can be attributed to a factor present in neoplasms which directly inhibits macrophage chemotactic responsiveness. In our studies, an allogeneic and three syngeneic neoplastic cell lines, as well as their soluble products, were tested for their effect on macrophage accumulation in vivo in C3H/HeJ mice and on macrophage or polymorphonuclear leukocyte (PMN) chemotactic responsiveness in vitro. The three syngeneic tumor lines were fibrosarcoma BP8, hepatoma 129, and lymphosarcoma 6C3HED. An allogeneic teratocarcinoma which developed in 129 mice was also used (*6*). Nonneoplastic cells were derived from normal syngeneic liver or spleen. Supernatants (1800*g* for 10 minutes) of centrifuged homogenates of neoplastic or control cells were made from sonicated washed neoplastic cells (5×10^7 cells per milliliter), or the same packed volume of control tissue, contained in 0.05*M* phosphate-buffered, *p*H 7.2, isotonic PBS saline. The dialyzable portions of the supernatants were collected after overnight dialysis (at 4°C) of 2.5 ml of the neoplastic or control tissue supernatants against 5.0 ml of RPMI 1640 medium (Grand Island).

We have already shown that the injection of phytohemagglutinin into the peritoneal cavities of mice produces a delayed-type inflammatory reaction characterized by the influx of macrophages, which reach peak numbers by 24 to 48 hours (*5*). To determine the effect of tumor implantation on macrophage accumulation in vivo, mice were given subcultaneous injections, in the thighs, of one of the neoplastic cell lines or control material, and 5 days later they were given an intraperitoneal injection of phytohemagglutinin (*7*). The implantation of the three syngeneic tumors inhibited macrophage accumulation in response to an intraperitoneal injection of phytohemagglutinin by 49 to 54 percent (Table 1). The implantation of equal amounts of normal liver or spleen cells had no significant effect on macrophage accumulation when compared to untreated mice. The administration of living cells of the allogeneic teratocarcinoma did not produce a tumor and did not depress the macrophage response.

To determine whether soluble factors contained in neoplastic cells could account for their inhibitory effect on macrophage accumulation, we injected subcutaneously in the thighs of C3H mice supernatant of sonicated tissues from fibrosarcoma BP8, hepatoma 129, lymphosarcoma 6C3HED, teratocarcinoma, or normal liver or spleen. Two days later the mice were injected intraperitoneally with phytohemagglutinin, and 48 hours later the numbers of macrophages that had accumulated in the peritoneal cavities were determined. Injection of the four different supernatants derived from malignant cells, including that of the allogeneic teratocarcinoma, depressed macrophage accumulation by 38 to 73 percent when compared to mice treated with no supernatants (Table 1). Injection of supernatants derived from normal tissues had no effect on macrophage accumulation. The initial characterization of the ihibitory activity by molecular sieve chromatography and pressure ultrafiltration indicated it was present in

Table 1. Inhibition of macrophage accumulation in vivo by neoplastic cells and their soluble products. The values represent the mean (± S.E.M.) number of macrophages recovered from the peritoneal cavities 2 days after intraperitoneal injection of 35 μg of phytohemagglutinin into five normal mice or five mice previously injected in the thigh with the indicated cells, supernatants, or dialyzates. The number of macrophages recovered from the peritoneal cavities of untreated mice was approximately 2×10^6 per mouse. Injection of the tumor products alone did not alter this value. Hence, the differences after phytohemagglutinin injection are due to macrophage accumulation. *R*, response; *I*, inhibition.

Source of cells	Number of macrophages ($\times 10^6$) in the peritoneal cavities of mice given					
	Intact cells*		Supernatant†		Dialyzate‡	
	R	*I* § (%)	*R*	*I* (%)	*R*	*I* (%)
Hepatoma 129	3.2 ± 0.3	54	4.1 ± 0.6	52	2.3 ± 0.3	66
Lymphoma 6C3HED	3.5 ± 0.3	49	5.3 ± 0.9	38	1.8 ± 0.3	73
Sarcoma BP8	3.4 ± 0.4	51	2.3 ± 0.3	73	3.8 ± 0.3	43
Teratocarcinoma	5.9 ± 0.5	14	5.0 ± 0.2	41	2.4 ± 0.3	64
Liver	7.1 ± 0.7	0	9.1 ± 1.3	0	6.6 ± 0.3	1
Spleen	6.2 ± 0.4	10	9.5 ± 0.8	0	6.7 ± 0.6	0
No cells	6.9 ± 0.6		8.5 ± 1.5		6.7 ± 0.4	

*Mice were injected subcutaneously with 2.5×10^6 of the indicated cells 7 days before they were killed. †The indicated tissues (5×10^7 cells per milliliter in PBS) were sonicated and centrifuged (1800*g* for 10 minutes); then 0.15 ml of the indicated supernatant fluid was injected subcutaneously in the thighs of groups of five C3H mice 4 days before they were killed. ‡Dialyzates were obtained by overnight dialysis of 2.5 ml of the supernatant of sonicated cells against 5 ml of RPMI 1640 and 0.2 ml of the indicated dialyzate injected in the thighs of groups of five C3H mice 3 days before they were killed. §$I(\%) = [1 - (R_E/R_c)] \times 100$, where R_E is the response of injected (with cells, sonicates, or dialyzates) mice and R_c is the response of the controls.

low-molecular-weight (< 10,000) fractions. To test the effect of these low-molecular-weight substances on macrophage accumulation in vivo, dialyzates of tumor or control supernatants were injected subcutaneously in the thighs of mice. While dialyzates of supernatants of normal cells had no effect on macrophage accumulation, dialyzates of the four neoplastic cell supernatants depressed macrophage accumulation in response to phytohemagglutinin by up to 73 percent.

To better define the mechanism by which tumor dialyzates depressed macrophage accumulation in vivo, the effect of these substances on the unidirectional migration of macrophages in vitro in response to chemotactic factors was measured (*8*). Peritoneal macrophages from normal mice were incubated with dialyzates from tumor or control tissues or with the dialyzate of medium alone and then tested for chemotactic responsiveness to endotoxin-activated mouse serum. The chemotactic responsiveness of macrophages incubated with tumor dialyzates was depressed by as much as 93 percent when compared to that of macrophages incubated with the dialyzate of medium alone (Table 2). Normal spleen or liver dialyzates had no significant effect on macrophage chemotaxis. When various amounts of the dialyzates were incubated with the chemotactic factor, the chemotactic response was depressed to a lesser extent than when the dialyzates were incubated with the macrophages, except in the case of fibrosarcoma BP8. In other studies, endotoxin-activated mouse serum, a chemotactic factor, was incubated with the dialyzate of the fibrosarcoma BP8 for 30 minutes at 37°C, and then dialyzed overnight to remove the tumor inhibitory factor. The serum treated in this manner retained the chemotactic activity for macrophages, thereby supporting the hypothesis that the tumor dialyzates do not irreversibly destroy the chemotactic factor.

The accumulation of PMN's in response to an intraperitoneal injection of endotoxin is not depressed in tumor-bearing mice (*5*). Isolated mouse peritoneal PMN's were incubated with the various dialyzates to determine their in vitro effect on PMN chemotactic responsiveness to activated serum (*9*) (Table 2). The dialyzates of neoplastic cells, which markedly depressed macrophage migration, had no significant effect on PMN chemotaxis, thereby demonstrating some specificity for the tumor products in affecting macrophages rather than affecting all inflammatory cells. To characterize the tumor-derived inhibitor of macrophage chemotaxis, we fractionated the sonicated supernatant of hepatoma 129 by chromatography on Sephadex G-50 (*10*). A sharp peak of inhibitory activity eluted at a volume corresponding to a molecular weight of approximately 6,000 to 10,000 (*11*).

Our studies demonstrate that malignant cells contain a low-molecular-weight factor capable of inhibiting macrophage accumulation in vivo and chemotactic responsiveness in vitro. The inhibitory factor depresses macrophage accumulation in vivo as dramatically as does a growing tumor. As might be expected, however, the effects of the isolated inhibitor are more transient than those of a viable tumor, the former lasting about 4 days, the latter lasting at least 12 days (*5*).

While the injection of viable allogeneic teratocarcinoma cells does not produce a tumor in C3H mice and did not depress macrophage function, the supernatant or dialyzate from the same allogeneic cells had inhibitory activity in vivo and in vitro. The amount of dialyzate injected was derived from approximately the same number of allogeneic cells which, when injected, caused no inhibition. Several possibilities could account for the inability of the whole allogeneic tumor cells to produce depression of macrophage accumulation in vivo: (i) only growing neoplastic cells may release the inhibitory factor in vivo, (ii) the allogeneic cells may be rejected before the factor can be released in vivo, or (iii) factors involved in the allograft rejection may overcome the effect of the inhibitor. Although the tumor-derived factor depresses macrophage chemotactic responsiveness in vitro, we have not eliminated the possibility that the inhibitor may also affect the activation or maturation of

Table 2. Inhibition of macrophage chemotaxis in vitro by dialyzates of sonicated neoplastic cells. *R*, response; *I*, inhibition.

Dialyzate*	Percent used	Dialyzates incubated with: Macrophages† *R*‖	Macrophages† *I*¶ (%)	Chemotactic factor‡ *R*‖	Chemotactic factor‡ *I*¶ (%)	PMN's§ *R*#	PMN's§ *I*¶ %
Lymphoma 6C3HED	50	5.0 ± 1.4	93	45.5 ± 5.2	31	356.7 ± 34.4	0
	30	18.8 ± 3.1	72	52.8 ± 2.1	23		
	10	38.3 ± 0.9	44	64.0 ± 4.0	4		
Hepatoma 129	50	39.0 ± 3.8	42	61.7 ± 3.6	6	349.8 ± 10.4	0
	30	48.5 ± 5.9	27	64.7 ± 4.0	5		
	10	62.4 ± 1.9	9	65.0 ± 1.2	2		
Teratocarcinoma	50	36.0 ± 2.1	47	58.7 ± 4.2	11	371.9 ± 12.3	0
	30	47.5 ± 2.1	28	65.3 ± 5.2	4		
	10	61.4 ± 3.6	10	64.0 ± 2.1	4		
Sarcoma BP8	50	31.0 ± 7.8	54	19.1 ± 1.9	71	372.6 ± 14.1	0
	30	31.4 ± 3.3	52	23.4 ± 2.1	66		
	10	44.9 ± 0.5	34	31.0 ± 2.6	54		
Normal spleen	50	70.3 ± 3.6	0	66.3 ± 1.6	0	361.7 ± 32.8	0
	30	65.0 ± 4.2	2	65.7 ± 1.9	4		
	10	68.0 ± 1.6	0	64.0 ± 1.4	4		
Normal liver	50	64.7 ± 1.2	4	63.7 ± 2.5	3		
	30	68.6 ± 4.7	0	65.7 ± 3.1	4		
	10	68.0 ± 1.4	0	67.7 ± 0.2	0		
Medium alone	50	67.7 ± 4.7		66.0 ± 1.9		373.2 ± 6.8	
	30	66.0 ± 2.9		68.3 ± 1.9			
	10	68.3 ± 5.9		66.7 ± 0.2			
No dialyzate**				63.7 ± 3.1		334.0 ± 22.9	
Negative control††				8.9 ± 2.1		5.3 ± 0.5	

*A sample (2.5 ml) of the indicated tumor cell supernatant, control cell supernatant, or medium alone was dialyzed overnight against 5 ml of RPMI 1640 medium, *p*H 7.0. The amount of dialyzate used in the chemotaxis assay is indicated by its final percent concentration (by volume) in medium containing either the leukocytes or the chemotactic factor. †Peritoneal macrophages (2.2×10^6/ml) from normal mice injected 2 days earlier with phytohemagglutinin were incubated for 30 minutes at 37°C with RPMI 1640 medium containing the indicated amount of the appropriate dialyzate and tested for chemotactic responsiveness to endotoxin-activated mouse serum (AMS). ‡AMS was incubated for 30 minutes at 37°C with RPMI 1640 medium containing the indicated amount of the appropriate dialyzate and tested for chemotactic activity for macrophages. §Peritoneal polymorphonuclear leukocytes (PMN's) (3.0×10^6/ml) from normal mice injected with 75 μg of *S. typhosa* endotoxin 18 hours before they were killed were incubated for 30 minutes at 37°C with RPMI 1640 medium containing the indicated amount of the appropriate dialyzate and tested for chemotactic responsiveness to AMS. ‖Chemotactic response is expressed as the average number of migrating macrophages per oil immersion field (× 1540) ± S.E.M. ¶$I(\%) = 1 - (C_{ED}/C_M) \times 100$, where C_{ED} is the chemotactic activity of cells or AMS incubated with experimental dialyzates and C_M is the chemotactic activity of cells or AMS incubated with the dialyzate of medium alone. #Chemotactic response is expressed as the average number of migrating PMN's per high power field (×780) ± S.E.M. **Macrophages or PMN's incubated with undialyzed RPMI 1640 medium and tested for chemotactic responsiveness to AMS. ††Macrophages or PMN's incubated with undialyzed RPMI 1640 medium and tested for response to RPMI 1640 medium alone.

mononuclear leukocytes into chemotactically responsive cells in vivo. The inhibitor does not, however, have the properties of a chemotactic factor inactivator such as the products of higher molecular weights (> 68,000) described by Bronza and Ward (*12*). It is possible, though, that the factor specified herein is similar to the low-molecular-weight, anti-inflammatory products described by Graham and Graham and by Fauve *et al.* (*13*).

The biological significance in tumorigenesis of a factor or factors contained in neoplasms which depress macrophage function remains to be determined. The inhibitory activity could, however, retard the ability of the immune system to rapidly mobilize sufficient numbers of macrophages to a developing tumor site to produce destruction of the neoplasm.

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6. The neoplastic cell lines were supplied by Drs. D. B. Amos and L. Gooding.
7. Two days after the intraperitoneal injection of 35 μg of purified phytohemagglutinin (Burroughs Wellcome), the mice were killed; the peritoneal cavities were exposed by abdominal incision and then lavaged vigorously with 10 ml of Gey's balanced salt solution containing 2 percent bovalbumin (Flow Laboratories), 0.01*M* HEPES buffer, *p*H 7.0 (Gey's BSS), and 10 units of heparin per milliliter to remove the accumulated inflammatory cells. The exudates from individual mice were centrifuged at 300*g* (4°C for 10 minutes) and resuspended in 1 ml of Gey's balanced salt solution; the total number and type of accumulated cells were determined (*5*).
8. In vitro chemotaxis was measured with peritoneal macrophages from normal mice that had been injected with phytohemagglutinin 48 hours earlier. The extensively washed cells were standardized to contain 2.2×10^6 macrophages per milliliter in medium RPMI 1640, and 0.4 ml of this suspension was placed in the upper compartment of a modified Boyden chamber. The cells were separated from the chemotactic stimulus, endotoxin-activated mouse serum, or medium alone by a 5.0-μm polycarbonate (Nuclepore) filter. All assays were performed in triplicate, and the chambers containing cells and stimulants were incubated for 4 hours in humidified air at 37°C. Chemotaxis was quantified by counting and averaging the number of macrophages per field in 20 (oil immersion) fields (×1540) that had migrated completely through the filter [R. Snyderman, M. C. Pike, D. McCarley, L. Lang, *Infect. Immun.* **11**, 488 (1975)].
9. Polymorphonuclear leukocytes were obtained from the peritoneal cavities of C3H/HeJ mice that had been injected with 75 μg of *Salmonella typhosa* 0901 endotoxin (Difco) 18 hours previously. The extensively washed PMN's (3.0×10^6 per milliliter of RPMI 1640) were placed in the upper compartment of a modified Boyden chamber and separated from the chemotactic stimulus, endotoxin-activated mouse serum, or medium alone by a 5.0-μm nitrocellulose (Millipore) filter. After incubation for 3 hours in humidified air, the filters were processed as described [R. Snyderman, J. K. Phillips, S. E. Mergenhagen, *J. Exp. Med.* **134**, 1131 (1971)].
10. The supernatant (3.0 ml) of sonicated hepatoma 129 cells was applied to a Sephadex G-50 (Pharmacia) column (2.5 by 55 cm) and eluted with RPMI 1640, *p*H 7.0, containing 0.01*M* HEPES buffer. Fractions were tested for inhibitory activity of macrophage chemotactic responsiveness.
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Growth Promotion by Homocysteic Acid

Abstract. *Homocysteic acid, $HO_3SCH_2CH_2CHNH_2CO_2H$, promotes growth of hypophysectomized rats, assayed by observation of increased thickness of epiphyseal cartilage of the tibia and by observation of tail growth. Doses of homocysteic acid as low as 1 microgram per day for 4 days in the tibia assay and 2.5 milligrams per kilogram per day for 5 weeks in the tail assay were effective in promoting growth. Serum somatomedin activity, determined by the porcine cartilage disk assay, was also increased by homocysteic acid. These findings relate an area of sulfur amino acid metabolism to the physiological action of growth hormone, accelerated growth in homocystinuria, initiation of arteriosclerosis, and control of cellular growth.*

Abnormalities of homocystine metabolism are associated with accelerated skeletal growth in individuals with homocystinuria (*1, 2*). Myointimal hyperplasia is observed both in individuals with homocystinuria (*3*) and in animals given homocystine derivatives (*4, 5*). We investigated the chemical nature of the homocystine derivative that promotes skeletal growth and hyperplasia of myointimal cells by assaying several sulfur amino acids for growth response in hypophysectomized rats. We found that homocysteic acid, the sulfonic acid derivative of homocysteine, promotes growth of hypophysectomized rats and that the serum from these animals contains somatomedin activity similar to that of normal rats. Thus the biological effects of homocysteic acid are similar to those produced by pituitary growth hormone in hypophysectomized rats. We believe these findings support the previous suggestion that homocystine derivatives are important in the physiological action of growth hormone and the initiation of arteriosclerosis (*6*).

Growth hormone activity was assayed by observation of tail growth in hypophysectomized rats given thyroxine, accord-

Table 1. Growth hormone activity of homocysteic acid (tail assay). Hypophysectomized rats (Charles River), tail length 135 to 150 mm, were injected 6 days per week with 6 μg of L-thyroxine subcutaneously and with sulfur amino acids in 0.01*M* sodium citrate, *p*H 8.9, intraperitoneally (*7*). Purified porcine growth hormone (Calbiochem) was used as a positive control. The last two groups were modified from data of de Groot (*7*). The *P* values were calculated for differences from the control group, by the paired *t*-test. Completeness of hypophysectomy was verified by dissection of the sella turcica.

Compound	Daily dose (mg/kg)	Animals		Days	Tail growth (cm)	*P*
		Number	Survivors			
None		20	14	33–37	0.9 ± 0.05*	
Homocysteic acid	2.5	11	5	35	2.0 ± 0.05	<.01
	20	10	4	33	1.9 ± 0.22	<.01
	80	18	8	33	2.5 ± 0.42	<.01
Homocystine	10	9	6	35	1.4 ± 0.28	>.1
Homolanthionine sulfone†	0.5	14	9	35	0.7 ± 0.06	>.8
Homolanthionine sulfone and sulfoxides†	0.5	14	13	35	0.6 ± 0.12	>.6
Growth hormone	1.0	10	4	37	2.0 ± 0.03	<.01
Growth hormone (*7*)	1.0	23	16	36	1.8	
None (*7*)		44	35	36	1.1	

*Means ± S.E.M. †Homolanthionine sulfoxide and homolanthionine sulfone were synthesized in our laboratory (*10*).