

Inhibition of Lung Tumor Colonization by Leech Salivary Gland Extracts from *Haementeria ghilianii*¹

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ABSTRACT

Salivary gland extract from the South American leech *Haementeria ghilianii*, administered i.v. on the same day as the i.v. inoculation of T241 sarcoma cells, completely suppresses colonization of the mediastinal lymph nodes and markedly reduces the number and size of lung tumor colonies produced by this tumor. Additional studies indicate that the extract contains various types of proteinase inhibitors and has the capacity to inhibit clotting and platelet aggregation by tumor material and collagen. Although not yet proved by direct evidence, these activities may be involved in the inhibitory effect of lung tumor colonization by the leech extract.

INTRODUCTION

Blood-sucking animals, such as leeches, mosquitoes, and vampire bats, have long been known to possess in their saliva substances which maintain the blood in a liquid state during the process of suction and subsequent storage in the gut. For this reason, leeches have been used for bloodletting since antiquity. Although this practice achieved great popularity in Europe at the beginning of the last century and has since been abandoned, interest in the nature and properties of leech anticoagulants has been renewed recently as a consequence of our greater understanding of the biochemistry of blood clotting and our search for new antithrombotic agents. These investigations have culminated in the isolation and characterization of several leech anticoagulants such as hirudin (13), a specific thrombin inhibitor from *Hirudo medicinalis*; hementerin (10), a plasminogen activator from *Haementeria lutzi*; and hementin (1, 2), a fibrinogen- and fibrin-degrading enzyme from *Haementeria ghilianii* (16, 17). Besides anticoagulants, the leech salivary gland contains many other constituents the function and nature of which are incompletely understood (2). Inhibitors of various proteases (4, 18)³ are among these compounds. Since administration of either anticoagulants (8, 20) or protease inhibitors (7, 15) from other sources has been reported previously to decrease tumor dissemination and metastasis, we investigated whether administration of crude *H. ghilianii* salivary gland extracts might affect the production of lung tumor colonies following the i.v. inoculation of murine sarcoma T241 cells. Our results clearly indicate that the salivary gland extract is a potent, nontoxic agent capable of

inhibiting the formation of metastases. The specific nature of the active fraction(s) and the mechanism(s) by which metastases are prevented are currently the subjects of intense inquiry.

MATERIALS AND METHODS

Tumor

Sarcoma T241 (12), a dimethylbenzanthracene-induced tumor, originally derived from a C57BL/6 mouse, was maintained as ascitic tumor in syngeneic C57BL/6 mice or C57BL/6 × A F₁ (hereafter called B6AF₁) compatible hybrids (The Jackson Laboratory, Bar Harbor, Maine). The capacity of its cells to induce platelet aggregation and coagulation was assayed as described previously (6). Addition of up to 2 × 10⁶ T241 cells to 500 μl of heparinized mouse or rat platelet-rich plasma (5 to 10 units of heparin per ml) failed to aggregate platelets. The tumor cells, however, did display a moderate procoagulant activity, as reflected by a shortened recalcified clotting time. The addition of 10⁶ T241 cells to syngeneic citrated plasma reduced the clotting time to between one-third and one-half of control levels. Another property of these cells is the capacity to degrade type IV collagen as reported by Liotta *et al.* (12).

Model of Metastasis

Lung tumor colonies were induced by the i.v. injection of T241 sarcoma cells. One-week-old ascitic tumor cells were separated by centrifugation (100 × g for 10 min) at 4°, washed twice with Hanks' solution, and suspended in the same medium (125,000 to 250,000 cells/ml). The suspension contained monodispersed cells of high viability (95%). Two-tenths ml of the suspension, containing 25,000 or 50,000 cells, was injected i.v. into male or female C57BL/6 mice (about 25 g) and into B6AF₁ mice (25 to 30 g), respectively. The animals, housed under identical conditions of temperature, photoperiodicity, and feeding, were killed 20 days later, and the number of metastases was counted with a Bausch & Lomb dissecting microscope.

Preparation of Crude Salivary Gland Extract

The salivary gland extract was obtained from the anterior glands of the *H. ghilianii* leech by a procedure described by Budzynski *et al.* (1, 2). Essentially, the dissected glands were freeze-dried, homogenized in pyrogen-free 0.15 M Tris-HCl buffer, pH 7.4, and centrifuged at 100,000 × g for 20 min. The resulting supernatant was then passed through a 0.22-μm pore filter (Millipore Corp., Bedford, Mass.). Any possible contamination of the extract by endotoxins has been excluded by tests of pyrogenicity in rabbits and by the negative dermal reaction of Shwartzman in the same animal.

Treatment of Mice with Salivary Gland Extract

Two-tenths ml of extract (400 μg of protein) was injected i.v. by the tail vein 2 hr before and 2 and 4 hr after the tumor cell inoculation. Controls were given similar injections but of pyrogen-free 0.15 M Tris-HCl, pH 7.4. Additional controls were treated with either one i.v. inoculation of 15 units of heparin (Elkins-Sinn, Cherry Hill, N. J.) or 2 units of *Bothrops atrox* venom (Atoxyn; Sigma Chemical Co., St. Louis, Mo.) in 0.2 ml of 0.9% NaCl solution given 1 hr before tumor inoculation.

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Assay of Protease Inhibitors in Crude Extract

Various amounts of crude extract were assayed for their ability to inhibit trypsin, plasmin, chymotrypsin, and granulocyte elastase using chromophoric or fluorescent substrates. A more detailed account of these inhibitory properties will be given in a separate paper.³ Reactions were allowed to proceed in the presence and absence of extract to determine the percentage of inhibition.

Trypsin Inhibitor Assay. After prewarming to 37°, 50 µl of a 40 µg/ml trypsin solution were added to a solution of 0.55 ml of buffer (0.1 M Tris-HCl buffer: 20 mM CaCl₂, pH 7.8), 0.2 ml of *N*-benzoyl-DL-arginine-*p*-nitroanilide [Sigma; H₂O (1 mg/ml)], and salivary gland extract. Ten min later, the reactions were stopped by the addition of 0.2 ml of 50% acetic acid and read in a spectrophotometer (Spectronic 710; Bausch & Lomb) at 405 nm against a blank which contained all of the above components except the enzyme.

Plasmin Inhibitor Assay. Fifty µl of porcine blood plasmin (Sigma; 40 µg of plasmin per ml of H₂O) was added to a 37° solution containing 0.55 ml of 0.05 M Tris-HCl buffer, pH 7.4, with 12 mM NaCl, 0.2 ml of Kabi substrate S2251 (AB Kabi, Greenwich, Conn.; 3 mM in H₂O), and salivary gland extract. After 10 min of incubation, 0.2 ml of 50% acetic acid was added, and the reactions were read as above.

Chymotrypsin Inhibitor Assays. To a 37° solution containing 0.35 ml of buffer (0.1 M Tris-HCl buffer, pH 8.3, with 0.96 M NaCl), 0.2 ml of the Kabi substrate S2586 (0.6 mM in H₂O), and salivary gland extract, 50 µl of chymotrypsin (400 ng/ml) were added. After 4 min, 0.2 ml of 50% acetic acid was added, and samples were read as above.

Elastase Inhibitor Assay. Inhibition of granulocyte elastase by salivary gland extract was kindly assayed by Dr. Harold James, Department of Medicine, Temple University, using the fluorometric method described by Zimmerman et al. (21). Elastase attack of the substrate *N*-tert-butoxycarbonyl-alanylprolylvalyl-4-methylcoumaryl amide split off 4-methylcoumaryl amide, and the fluorescence emitted by this free group is recorded by an Amicon-Bowman spectrofluorometer.

Study of the Anticoagulant Activity of the Salivary Gland Extract

C57BL/6J male mice were given i.v. injections of 0.2 ml of salivary gland extract (2 mg/ml of protein) or 0.2 ml of Tris-HCl buffer, pH 7.4, and bled from the heart 2, 4, and 6 hr after treatment. Nine parts of blood were mixed with 1 part of 3.2% sodium citrate, and plasma from each mouse was separated by centrifugation in an Eppendorf ultracentrifuge (8000 × *g* for 2 min). Recalcified and thrombin-clotting times were performed by adding to aliquots of 0.1 ml of plasma, prewarmed at 37° for 3 min, 0.1 ml of 25 mM CaCl₂, and 0.1 ml of thrombin solution, respectively. The thrombin of human origin (Sigma) was reconstituted with water and diluted 1:1 with 0.9% NaCl solution.

Assay of the Antiplatelet Activity of the Salivary Gland Extract

B6AF₁ female mice were given i.v. injections of 0.2 ml of salivary gland extract (2 mg/ml of protein) or 0.2 ml Tris-HCl buffer, pH 7.4, and bled from the heart 2, 4, and 6 hr after treatment. Platelet-rich plasma was prepared from heparin-anticoagulated blood (5 units/ml), and the response of the platelets to tumor material was determined in an aggregometer by adding 50 µl of plasma membrane vesicles (50 µg of protein) from 15091A tumor cells to 450 µl of platelet-rich plasma. The activity of the extract against platelet aggregation induced by collagen was tested *in vitro* by using heparinized rat platelet-rich plasma (10 units/ml of heparin) and acid-soluble collagen prepared from bovine Achilles tendon (Worthington Biochemical Corp., Freehold, N. J.). For this purpose, 400 µl of platelet-rich plasma were incubated at 37° with 50 µl of salivary gland extract (2 mg/ml of protein). After 20 min of incubation, 50 µl of various dilutions of collagen in saline were added, and platelet aggregation was recorded with a Payton aggregometer (Buffalo, N. Y.).

Statistical Analyses

The difference in the percentage of mice with mediastinal tumors and in the number of lung tumors between tested groups was analyzed with the χ^2 and Mann-Whitney tests, respectively.

RESULTS

Inhibitory Activity of the Salivary Gland Extract against Proteolytic Enzymes. The performed assays demonstrated the presence of inhibitors against trypsin, plasmin, chymotrypsin, and granulocyte elastase. To produce 50% inhibition of each enzyme activity, the average amounts of extract needed were 35, 9, 23, and 3 µg of protein, respectively. A minimum of 3 to 5 assays were performed with each enzyme.

Inhibitory Effect of the Salivary Gland Extract on Coagulation and Platelet Aggregation. The *in vivo* administration of the extract prolonged both the thrombin and the recalcified clotting times, with a greater effect on the latter. When blood was drawn at 2, 4, and 6 hr after treatment (Table 1), the extract also inhibited platelet aggregation induced by plasma membrane vesicles shed by the murine mammary adenocarcinoma 15091A, an effect that lasted for 2 to 4 hr after i.v. administration of the extract. While the platelets of untreated rat controls responded with a typical aggregation to the various doses of collagen (lag period, from 0.2 to 0.6 min), platelets incubated with the extract were totally unresponsive to the same aggregation stimulus.

Inhibitory Effect of the Salivary Gland Extract on Lung Tumor Colonies. I.v. injection of T241 sarcoma cells into syngeneic or compatible hybrid mice regularly produced numerous lung tumor colonies, and neoplastic involvement of the mediastinal lymph nodes. No metastases were observed in other organs. Three i.v. injections of the salivary gland extract, given to C57BL/6J mice on the same day as tumor inoculation, reduces the percentage of animals developing mediastinal and lung tumors from 100 to 0 and 100 to 45, respectively.

Furthermore, those mice that do develop pulmonary metastases have far fewer metastases, and their tumors are significantly reduced in size (Table 2). The leech extract treatment was equally effective in B6F₁ mice. In this group of animals, 3 i.v. injections of extract totally suppressed mediastinal lymph node metastases and dramatically reduced the median number of lung tumor colonies per mouse from 61 to 3 (Table 2). Likewise, the average diameter of the lung tumor colonies was diminished to one-half of that of the untreated group.

Table 1

Effect of leech salivary gland extract on clotting and platelet aggregation
See "Materials and Methods" for additional details.

In vivo treatment	Treatment time (hr)	Recalcified clotting time (sec)	Thrombin clotting time (sec)	Platelet aggregation	
				Lag (min)	% of aggregation
Buffer	2	99 ± 4 ^a	30 ± 1	2.2 ± 0.2	72 ± 3
Extract	2	167 ± 3	29 ± 1		0
Buffer	4	67 ± 2	26 ± 1	3.4 ± 0.3	78 ± 2
Extract	4	Unclottable ^b	240 ± 10		0
Buffer	6	53 ± 2	26 ± 1	2.8 ± 0.2	80 ± 4
Extract	6	Unclottable ^b	198 ± 5	2.3 ± 0.1	70 ± 2

^a Mean ± S.D.

^b More than 1 hr.

Table 2
Effect of leech salivary gland extract on lung and mediastinal colonization produced by i.v. injection of T241 sarcoma cells

Treatment	% of mice with tumors		Lung tumors/ mouse ^a		<i>p</i> ^b	<i>p</i> ^c	Diameter of lung tumors ^e (mm)
	Mediastinal	Lung	Median	Range			
C57BL/6J mice							
Control (Tris-HCl buffer)	100	100 (30) ^d	8	1-25			2.3 ± 0.1 ^e
Salivary gland extract	0	47 (30)	2	1-3	0.001	0.001	1.4 ± 0.1
Heparin	80	100 (10)	5	5-8	NS ^f	NS	2.2 ± 0.1
Atroxin	70	100 (10)	4	1-10	NS	0.05	2.2 ± 0.3
B6AF ₁ mice							
Control (Tris-HCl buffer)	100	100 (13)	61	22-87			2.7 ± 0.2
Salivary gland extract	0	100 (10)	3	1-9	0.001	0.001	1.3 ± 0.1

^a Excluding mice without lung tumors.

^b Probability of no difference, in percentage of mice with mediastinal tumors, between treated and control mice.

^c Probability of no difference, in number of lung tumors, between treated and control mice.

^d Numbers in parentheses, number of mice per group.

^e Mean ± S.E.

^f NS, not significant.

DISCUSSION

Our preliminary research indicates that salivary gland extract, a complex mixture of active compounds (2), has the capacity to markedly reduce the number of metastatic tumor colonies induced by i.v. injection of T241 sarcoma cells. Since the hemostatic system has been thought to contribute to metastasis formation by causing vascular arrest of tumor cells (5, 8, 9, 14, 19, 20), the question that arises is whether it is the capacity of the extract to inhibit coagulation, platelet aggregation, or both, that is the responsible mechanism for the dramatic antimetastatic effect demonstrated. Although T241 sarcoma cells lack the ability to aggregate platelets *in vitro* directly (see "Materials and Methods"), platelet aggregation may be induced by exposed collagenous components of the basement membrane and underlying connective stroma, following attack on the vascular lining by T241 sarcoma cells (12). This possibility has been shown to occur *in vitro* at least in the case of rat platelets exposed to bovine collagen. Hence, the possibility still remains that inhibition of platelet aggregation by the extract contributes to the inhibitory effect of the extract on metastasis.

To find out whether the inhibition of metastasis observed can be fully explained by the anticoagulant activity of the extract, mice were also treated with conventional anticoagulants. The use of heparin alone, which acts by potentiating antithrombin III, or Atroxin, which, like hementin, is another fibrinolytic agent, reduced only slightly the number of lung and mediastinal tumors (Table 2). Even when heparin was given at much higher doses (50 units i.v. or i.p.) and more frequently, the inhibitory effects on metastasis seen with the extract could not be reproduced (data not shown).

Hence, these results suggest that other or additional mechanisms must underlie the capacity of the extract to inhibit metastases. Such a contributory mechanism may be the inhibition of tumor cell extravasation by the extract. Fidler *et al.* (3), and Liotta *et al.* (11, 12) have reported that the crossing of anatomical barriers such as epithelial cell and vascular basement membranes by tumor cells first requires proteolysis of these structures by the invading cells. Since the crude extract contains various types of protease inhibitors,³ the possibility exists that these inhibitors may block the basement membrane-degrading

capacity of the tumor cells, thus preventing tumor cell extravasation and metastasis formation.

One striking effect of salivary gland extract administration is the total suppression of neoplastic involvement of the mediastinum which is invariably present in control mice. This result suggests that the extract is capable of inhibiting invasion of the lung lymphatics after sarcoma cells have escaped from the blood circulation or that the extract can block colonization of the mediastinal lymph nodes by a mechanism still to be determined.

Work is in progress to fractionate our extract; to test each fraction for its capacity to inhibit coagulation, platelet aggregation, and proteolysis of basement membrane components; and also to evaluate the ability of each fraction to inhibit metastasis in the mouse model described.

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