

Production of a Low Molecular Weight Eosinophil Polymorphonuclear Leukocyte Chemotactic Factor by Anaplastic Squamous Cell Carcinomas of Human Lung

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ABSTRACT A peptide of approximately 300–400 daltons exhibiting in vitro chemotactic activity for human polymorphonuclear (PMN) leukocytes, with a preference for the eosinophil series, was isolated from extracts of anaplastic lung carcinomas of the large squamous cell type obtained from three patients with marked peripheral blood hypereosinophilia and eosinophilic infiltration of the tumors and surrounding normal pulmonary tissues. This chemotactic factor was termed ECF-LSC (eosinophil chemotactic factor of lung squamous cell carcinoma). ECF-LSC appeared in the urine of two of the patients in increasing quantities late in the course of their disease and was also elaborated by long-term cultures of dispersed tumor cells from the same two patients. Three anaplastic large cell bronchogenic carcinomas which were not associated with tumor tissue or peripheral blood eosinophilia, a bronchogenic adenocarcinoma from a patient with only peripheral eosinophilia, and a renal cell carcinoma metastatic to the lungs and associated with transient pleural tissue and fluid eosinophilia were all devoid of ECF-LSC. ECF-LSC from tumor tissue extracts, urine, and tumor cell culture medium was comparable to the mast cell-associated tetrapeptides of the eosinophil chemotactic factor of anaphylaxis (ECF-A) in size, but eluted from Dowex-1 at pH 5.0–3.5 in contrast to the more acidic ECF-A tetrapeptides which eluted at pH 3.2–2.2. ECF-LSC, like the tetrapeptides of ECF-A, had a secondary chemotactic activity for neutrophil PMN leukocytes, but not

mononuclear leukocytes, and deactivated both eosinophil and neutrophil PMN leukocytes so that they would not respond to a subsequent in vitro chemotactic stimulus. Eosinophils from the two patients with urinary excretion of ECF-LSC and the highest concentrations in tumor extracts were hyporesponsive in vitro to homologous and heterologous chemotactic stimuli, suggesting that ECF-LSC had deactivated the eosinophils in vivo.

INTRODUCTION

The appearance of peripheral blood eosinophilia in association with a malignant tumor is an uncommon occurrence and usually signifies either the development of metastatic lesions or a tissue response to irradiation of the tumor (1–3). In a few cases, however, peripheral eosinophilia has resolved upon removal of the neoplasm which implies an etiologic relationship to the tumor (1, 4). In one patient with an anaplastic bronchogenic carcinoma associated with both peripheral eosinophilia and eosinophilic infiltration of the tumor, a low molecular weight peptide chemotactic for eosinophils was extracted from tumor tissue (5). The tumor-derived eosinophil chemotactic factor (5) was comparable to the tetrapeptides of the mast cell-derived eosinophil chemotactic factor of anaphylaxis (ECF-A)¹ (6) with respect to size, function, and susceptibility to inactivation by digestion with subtilisin. The tumor

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¹ Abbreviations used in this paper: DCC-trypsin, trypsin previously reacted with diphenyl carbamyl chloride; ECF, eosinophil chemotactic factor; ECF-A ECF of anaphylaxis; ECF-LSC, ECF of lung squamous cell carcinoma; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; hpf, high power field; MEM, Eagles' Minimal Essential Medium with Earle's salts; PMN, polymorphonuclear.

cells were implicated as the source of the chemotactic principle by the finding of clusters of eosinophils around free tumor cells in the pleural space and the absence of detectable mast cell constituents such as histamine in tumor extracts (5). The identification of two additional patients with marked local and peripheral eosinophilia in association with anaplastic bronchogenic carcinoma provided the opportunity to establish by *in vitro* culture techniques that the tumor cells were the source of eosinophilotactic peptide. The tumor-associated peptide, derived by direct extraction of tumor tissue or cell culture medium, was comparable in size but less acidic than the ECF-A tetrapeptides, which implies a difference in the charged residues of the amino acid composition.

METHODS

Materials. Hanks' balanced salt solution with phenol red (Microbiological Associates, Walkersville, Md.), sodium diatrizoate (Hypaque, Winthrop Laboratories, New York), dextran, Sephadex G-25, Ficoll, and chromatography columns (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), Dowex AG-1-X8, 200–400 mesh (Bio-Rad Laboratories, Richmond, Calif.), ovalbumin five times recrystallized (Miles Laboratories, Inc., Elkhart, Ind.), trypsin previously reacted with diphenyl carbamyl chloride (DCC-trypsin) and recrystallized (Sigma Chemical Co., St. Louis, Mo.), subtilisin (Miles-Yeda, Ltd., Rehoveth, Israel) which was recrystallized three times before use and its activity standardized (7), Eagles' Minimal Essential Medium with Earle's salts (MEM), horse serum and fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), 50-mm diameter plastic tissue culture dishes, and 50-ml and 250-ml plastic culture flasks (Falcon Plastics, Oxnard, Calif.), were obtained as noted. All solvents were reagent grade (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.) or Fisher-certified (Fisher Scientific Co., Pittsburgh, Pa.) and were redistilled before use. Histamine levels in tumor tissue and cell extracts and portions of chromatography fractions were measured by a radioenzyme assay (8).

Extraction and purification of chemotactic factors in tumor tissue, biologic fluids, and tumor cell culture medium. Surgical or autopsy specimens of human tumor tissues were processed to 10–20-mg fragments by mincing between two scalpel blades in Tyrode's buffer at 4°C. Portions of the fragments were extracted twice at 4°C with 2 ml per g of tissue of either butanol:glacial acetic acid (10:1, vol:vol), butanol:pyridine (10:1, vol:vol) or Tyrode's buffer-0.05 M Tris-HCl (pH 8.2) by homogenization with a tissue grinder (Polytron, Brinkmann Instruments, Inc., Westbury, N. Y.) and sonication for 15 min in a pulsed mode (Branson Sonic Power Co., Danbury, Conn.). The extracts from each tissue and solvent system source were pooled and centrifuged at 1,000 g for 20 min at 4°C to obtain a clear supernatant fraction.

Other portions of the fragments were minced with scissors under sterile conditions in MEM followed by treatment with 0.1% pancreatin solution (Viokase, VioBin Corp., Monticello, Ill.) for 5–8 min at 37°C (9) to obtain dispersed cells which were separated from residual tissue fragments by low speed centrifugation. The dispersed cells were washed with MEM and then plated in plastic Petri dishes or flasks in MEM containing 5% fetal calf serum and 5% horse serum (9). Cultures were incubated at 37°C in humidified 5% CO₂-95%

air, and medium was harvested and renewed once weekly in flasks and twice a week in dishes. 20–40 separate culture vessels were prepared from each tumor. After 2 wk of incubation, most vessels contained mixtures of epithelial-like and fibroblastic cells in variable proportions. Vessels containing over 90% epithelial-like cells were washed and propagated serially by subculture (9). Multiple attempts to establish clones derived from single cells were unsuccessful. Supernatant fluids were harvested from vessels containing predominantly epithelial-like cells, from those containing predominantly fibroblastic cultures, and from control cultures of normal diploid human fibroblasts. These samples were collected separately, or, on occasion, samples from the same line of cultures were pooled and stored at –70°C.

Supernatant fractions from cell cultures and tissue extracts were concentrated by lyophilization, dissolved in a volume of 0.1 M acetic acid equal to 1/10 that of the original volume of supernate, and applied to a Sephadex G-25 column. Urine and pleural fluid samples were applied to a Sephadex G-25 column directly or after concentration and enrichment for low molecular weight factors by pressure ultrafiltration (Amicon Corp., Lexington, Mass.) employing UM-10 and UM-05 membranes in sequence to retain molecules of molecular weight approximately 300 or greater. 80 × 1.5-cm Sephadex G-25 columns of 142-ml bed volume were equilibrated and eluted with 0.01 M acetic acid at 4°C employing a capillary pump (Gilson Medical Electronics, Inc., Middleton, Wis.) that produced flow rates of 3% bed volume per hour. Dowex AG-1 ion exchange chromatography employed a 0.1-M pyridine-0.5-M formic acid elution system which provided a linear gradient of –0.3 pH units per 4 bed volumes in the pH range from 5 to 2 (6). High voltage paper electrophoresis was performed with Whatman 3 MM paper (W & R Balston, Ltd., Kent, England) held between two sheets of plastic film over a horizontal aluminum cooling block maintained at 10°C (FP-30A, Savant Instruments, Inc., Hicksville, N. Y.). The buffer was 0.05 M pyridine-acetate (pH 5.6) and electrophoresis was carried out with 3,800 V over 75 cm for 4 h. 1-cm strips were eluted sequentially with 0.01 M acetic acid, distilled water, and 0.01 M ammonium hydroxide. The eluates for each strip were pooled and re-lyophilized twice from distilled water before chemotactic assays. Enzymatic treatment of replicate crude samples or partially purified fractions containing eosinophil chemotactic activity of 60–80 eosinophils per hpv was carried out for 6 h at 37°C with 1–10 μg of DCC-trypsin in 0.1 M Tris-HCl (pH 8) containing 0.01 M CaCl₂ or with 0.2–10 μg of subtilisin in 0.1 M ammonium acetate (pH 7.4).

Measurement of chemotaxis. Blood from normal subjects or patients with peripheral blood hypereosinophilia of 27–84% as a result of allergic or collagen-vascular diseases, drug reactions, or chronic active hepatitis was collected in tubes containing citrate anticoagulant and dextran at a final concentration of 1% (10). After a 30-min incubation at 37°C, the leukocyte-rich plasma was transferred to fresh tubes and centrifuged to obtain a cell pellet which was resuspended in 0.84% NH₄Cl for 2 min at room temperature to lyse residual erythrocytes. The mixed leukocytes were washed and suspended in Hanks' solution made 0.4 g/100 ml in ovalbumin and 0.01 M in Tris-HCl, pH 7.4 (Hanks'-ovalbumin). The suspensions of leukocytes from normal subjects were centrifuged on Ficoll-Hypaque cushions to obtain purified neutrophils and mononuclear leukocytes (11) and the suspensions of leukocytes from hypereosinophilic donors were used directly in chemotactic assays.

Chemotaxis was assessed by a modification (10, 12) of the Boyden micropore filter technique (13). 1 ml of cell suspension containing $2.0 \pm 0.5 \times 10^6$ eosinophils or neutrophils or

$3.5 \pm 0.3 \times 10^6$ mononuclear leukocytes was pipetted into the top compartment of each polystyrene disposable chemotactic chamber (Adaps, Inc., Dedham, Mass.); a 3- μ m pore filter (Millipore Corp., Bedford, Mass.) separated the leukocytes from the 1 ml of chemotactic stimulus in the lower compartment. After a 2.5-h incubation at 37°C, the filters were removed, processed, and stained by a procedure designed to differentiate eosinophils from neutrophils (10, 12), and the leukocytes were microscopically enumerated in 10 high power fields at a fixed level of 70–90 μ m from the cell source. This distance was selected to achieve background counts of two–eight leukocytes per high power field (hpf) in the absence of a chemotactic stimulus. Cells were then counted without knowledge of the protocol in 5 hpf from each of duplicate filters, and these values expressed as the mean net leukocytes per hpf by correction for spontaneous migration in additional control chambers. The chemotactic activity of pooled column fractions of tumor or other patient materials was calculated from the mean activity of the lowest dilution of the pool achieving a maximum response and the overall dilution, and was expressed as eosinophils/hpf per milligram of tissue or milliliter of fluid. Replicate determinations fell within the range of $\pm 35\%$ of the mean of all values which includes the

variation in eosinophil donors. The reduced responsiveness of leukocytes preincubated with a chemotactic stimulus for 30 min at 37°C, termed functional deactivation, was calculated as: $(1 - \text{chemotactic response of treated leukocytes/chemotactic response of control leukocytes}) \times 100$ (14–16). C5 fragments were prepared by tryptic digestion of purified human C5 (17). Kallikrein was purified from human serum by a three-column procedure and quantitated by bioassay of bradykinin generated from plasma kininogen (18). 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) was produced by the action of a platelet lipoxygenase on arachidonic acid in the presence of indomethacin and purified by silicic acid column chromatography (19).

RESULTS

Four patients with bronchogenic carcinoma and one patient with renal cell carcinoma metastatic to the lungs exhibited local and/or peripheral blood eosinophilia as a manifestation of their disease (Table I). Three of the patients (Table I, patients 1,2,3) who had anaplastic

TABLE I
Characteristics of Patients with Pulmonary Carcinoma and Eosinophilia

Patient	Sex	Age	Duration of disease	Primary site	Histology (EM)*	Spread of tumor	Therapy	Number and distribution of eosinophils				
								Peripheral blood†	Bone marrow‡	Primary tumor§	Metastases	Pleural fluid¶
		yr	wk									
1¶	F	53	11	Lung	Large cell, anaplastic (squamous type)	Lungs, pleura, pericardium, ribs, hilar lymph nodes	Prednisone	20,880–93,375	45%	+	+	+
2	M	63	15	Lung	Large cell, anaplastic (squamous type)	Lungs, pleura, pericardium	Dexamethasone	7,930–11,765	28%	+	+	+
3	M	69	60	Lung	Large cell, anaplastic, (squamous type)	Lungs, pleura, pericardium, para-aortic lymph nodes	Cyclophosphamide, Adriamycin	750–55,360	65%	+	+	+
4	M	65	12	Lung	Anaplastic adenocarcinoma, foci of cuboidal alveolar cells	Lungs	No chemotherapy	210–1,680	3%	0	0	–
5	M	65	58	Kidney	Renal clear cell	Widespread including lungs, pleura and pericardium, and bone marrow	Mithramycin	150–260	—	0	0	+

* Electron microscopic (EM) characteristics of tumor cells are noted in parentheses.

† Values are ranges of total eosinophil counts during the clinical course; normal range = 150–300 per mm³.

‡ Peripheral blood eosinophil counts were 56,540, 7,930, 14,800, and 1180, respectively, when the bone marrow samples were obtained from patients 1 to 4.

§ Eosinophils were present in abnormal numbers in tissue or fluid = +, or were absent = 0; not done = –.

¶ Patient 1 was previously reported (5).

bronchogenic tumors of the squamous cell type, with local spread throughout the chest, developed extreme levels of bone marrow and peripheral blood hypereosinophilia associated with eosinophilic infiltration of the tumor and surrounding normal lung. A fourth patient (Table I, patient 4) who had a primary bronchogenic adenocarcinoma showed transient peripheral blood eosinophilia without any eosinophil infiltration of tissues. A patient with renal cell carcinoma and widespread metastases (Table I, patient 5) had local eosinophilia in the pleural tissues and fluid, presumably in relation to spread of tumor into the lungs. Other known causes of eosinophilia such as parasitic infestations, atopy, drug reactions, and collagen-vascular disease were excluded in each case (20-22). Three patients, two men and one woman ranging in age from 52 to 71 yr, with large cell anaplastic lung tumors, but lacking tumor tissue or peripheral eosinophilia, were also studied.

Distribution of low molecular weight eosinophil

chemotactic factors in tumor tissue and body fluids from patients with lung tumors. Extraction of tumor tissue from patients 2 and 3 (Table I) in Tyrode's solution buffered with 0.05 M Tris-HCl (pH 8.2) provided quantities of eosinophil chemotactic activity (5610,6980 eosinophils per hpf per g) comparable to extraction in butanol-glacial acetic acid, 10:1 vol:vol (3370,4290 eosinophils per hpf per g) and exceeding extraction in butanol-pyridine, 10:1 vol:vol (1140,1630 eosinophils per hpf per g). As Tris-buffered Tyrode's had been utilized for earlier extraction of tumor tissue from patient 1 (5), it was adopted as the standard extraction buffer.

Filtration of extracts from the five tumors of patients with eosinophilia revealed multiple peaks of polymorphonuclear (PMN) leukocyte chemotactic activity (Fig. 1). All tumor tissue extracts possessed a high molecular weight peak in the column exclusion volume which was preferentially chemotactic for neutrophils and an intermediate peak containing factors of mol wt 1,500-

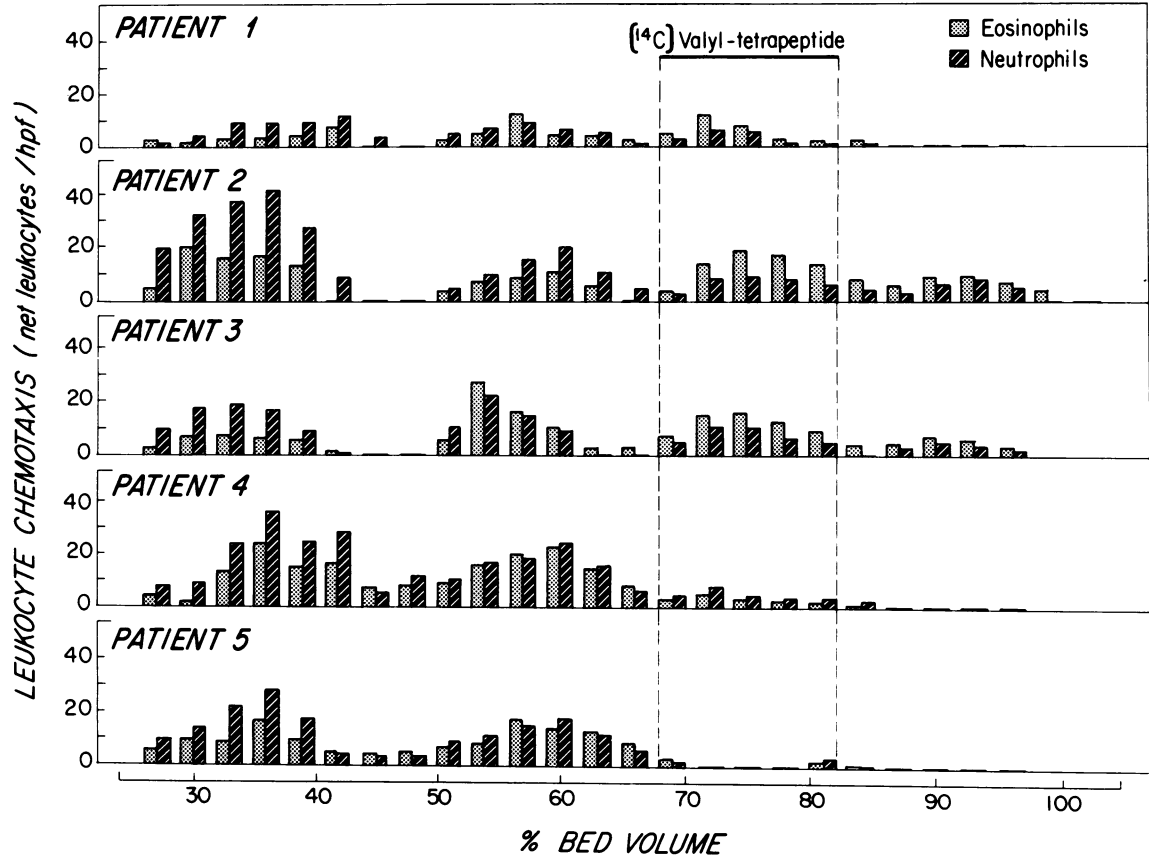


FIGURE 1 Sephadex G-25 filtration of extracts of tumor tissue. Extracts from 1 g wet weight of each tumor were applied to a Sephadex G-25 column, 1.42-ml fraction volumes were collected, and 0.1 ml was assessed in duplicate for eosinophil and neutrophil chemotactic activity. Neutrophils of 97% purity exhibited a background response of 3.6 cells/hpf while unpurified eosinophils from a donor with 44% eosinophilia had a background response of 4.8 cells/hpf.

3,000 daltons, which was about equally chemotactic for eosinophils and neutrophils. Tumor extracts from patients 1 to 3 exhibited a low molecular weight peak, attracting more than 10 net eosinophils/hpf per 100 μ l of each fraction, which filtered in the same region as the radiolabeled valyl-tetrapeptide of ECF-A and was chemotactic for PMN leukocytes, with a preference for eosinophils (Fig. 1). Crude extracts from lymph node tumor metastases, concentrated samples of a 24-h urine collection, and tumor cell culture fluid of patient 2 were filtered on Sephadex G-25 columns in an identical manner (Fig. 2). In the urine and culture fluid samples, the high molecular weight chemotactic activities were less prominent relative to the activities in the intermediate and low molecular weight regions, both of which exhibited preferential chemotactic activity for eosinophils over neutrophils. Because the low molecular weight peak of eosinophilotactic activity was present in samples from diverse sources of the patients with squamous cell lung tumors, and most closely resembled ECF-A tetrapeptides in size and preferential chemotactic activity for eosinophils compared with neutrophils and monocytes, it was selected for further analysis.

To provide a quantitative estimate of the low molecular weight eosinophilotactic activity from diverse sources, the fractions from 70–84% bed volume obtained from the tumor, urine, and culture fluid from patient 2 (Fig. 2) were lyophilized and resuspended in 2 ml of Hanks' solution. Eosinophil chemotactic activity was assessed at 5, 10, 25, and 50 μ l, each diluted to a final volume of 1 ml in the stimulus compartment. The net eosinophils attracted per hpf equaled 8, 17, 39, and 48 for the tumor extract; 6, 19, 32, and 30 for the urine concentrates; and 12, 23, 41, and 45 for the tumor cell culture medium. Since increasing concentrations of eosinophil chemotactic factor (ECF) from the three sources approached a plateau of eosinophil chemotactic activity, the content of low molecular weight ECF was estimated by multiplying the maximum eosinophil response by the greatest dilution achieving that response. With each source of material, the introduction of an equal dilution of stimulus on both sides of the filter in the chemotactic chambers eliminated the cellular response, indicating its dependence on a concentration gradient.

In the three patients with a squamous cell bronchogenic carcinoma, quantitation of the low molecular weight ECF in biopsies and autopsy samples of tumor (Table II, patients 1–3) revealed concentrations of 2 to 35 times the mean level of nontumorous human lung. In contrast, the histamine levels in the tumor, attributed to tissue mast cells, were 1/5 to less than 1/100 the mean concentrations in surgical specimens of human lung. Low molecular weight ECF was also present in substantial quantities in the urine of two of the patients

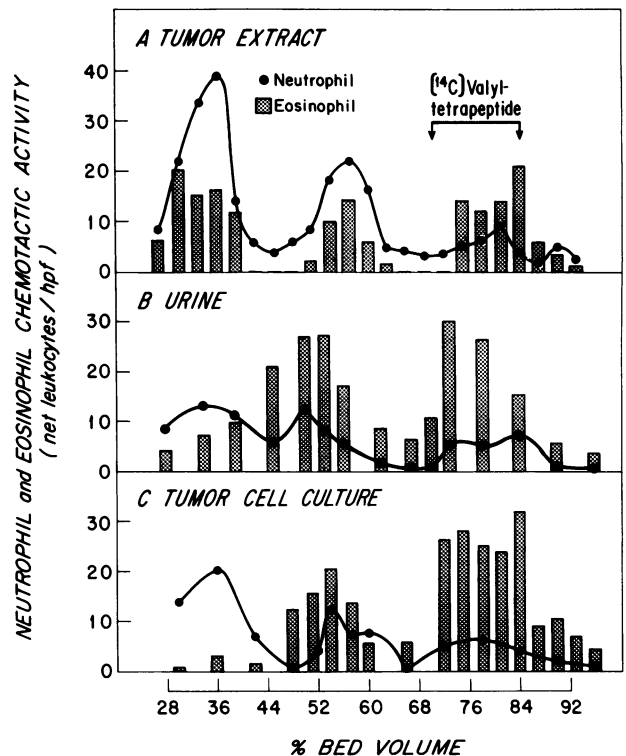


FIGURE 2 Sephadex G-25 filtration of extracts of tumor tissue, urine, and tumor cell culture medium from patient 2 (Table I). An extract of 1 g of tumor (A), a concentrate of one-half of a 24-h urine collection (B), and a concentrate of 10 ml of culture medium from epithelial-like cells obtained 3 mo after establishment in culture (C) were each redissolved in 2 ml of 0.01 N acetic acid and filtered on identical Sephadex G-25 columns. Fraction volumes of 4.3 ml were collected and 0.1-ml portions were assayed in duplicate for eosinophil and neutrophil chemotactic activity utilizing mixed leukocytes from a patient with 41% eosinophils and 34% neutrophils. Control fresh culture medium, conditioned medium from normal human fibroblasts and normal urine had no eosinophil chemotactic activity detectable in this dose range.

with squamous cell carcinoma and increased with progression of clinical disease (Table II, patients 2,3). The peripheral eosinophil counts had reached a plateau for patient 2 by wk 10 and for patient 3 by wk 43 and did not increase further with the rising urine level of the low molecular weight eosinophil chemotactic factor. The factor was not found in analyses of urine specimens from over 10 normal subjects. Furthermore, five separate subcultures of cells grown from tumors of patients 2 and 3 secreted low molecular weight ECF into the media which was not found in media from cultures of normal diploid human fibroblasts. Low molecular weight ECF falling within the concentration range observed in nontumorous human lung of 0.06–0.3 eosinophils/hpf per mg was identified in the tumor from patient 4, who had a bronchogenic adenocarcinoma (Table II). The anaplas-

TABLE II
Distribution of Eosinophil Chemotactic Factors and Chemotactic Responsiveness of Patient Eosinophils

Patient	Low molecular weight ECF activity*					Eosinophil migration				
	Primary tumor†	Lymph node or other metastasis	Pleural fluid	Urine	Tumor cell culture medium supernate	Tumor histamine‡	Random migration§	Chemotaxis°		Ascorbate enhancement of random migration¶
								Fragment of C5	Tumor ECF	
	per mg	per mg	per ml	per 24 h	per ml	ng/mg				%
1	0.4-B 1.0-T	—	—	—	—	0.07	4.8	31.6	24.3; 8 wk**	—
2	4.6-T	5.3-N	—	810; 10 wks** 2,160; 14 wks	480–690	0.12	5.1	0	0 ; 8 wk	20
3	6.4-B 1.1-T	—	—	200; 43 wks 1,045; 52 wks 8,382; 56 wks	290–410	1.82	3.2 4.6	24.9 11.2	15.2; 43 wk 6.8; 52 wk	146 104
4	0.2-B	—	—	<200	<50	2.56	4.1 3.6	27.4 28.1	3.5; 8 wk 2.7; 10 wk	161 155
5	—	0 (lung and femur)	390	<200	<50	0.08 (femur)				

* Eosinophil chemotactic activity of Sephadex G-25 low molecular weight pools are expressed as mean net eosinophils/hpf per unit of weight, volume, or time as calculated from the activity of the lowest dilution of each sample eliciting a maximum response and the dilution factor; 0 = no activity detectable; — = not available or not assayed; B = biopsy specimen; T = autopsy tumor; N = lymph node biopsy.

† Analyses of Sephadex G-25 low molecular weight pools of extracts of surgical samples of a normal portion of five lungs removed during surgery gave a mean value of 0.18 (0.06–0.3 range) eosinophils/hpf per mg while extracts of portions of the same lungs had a mean value of 8.4 (6.3–11.7 range) ng histamine per mg.

§ Mean eosinophils/hpf in the absence of a stimulus.

° Mean net eosinophils/hpf; C5 fragment was prepared by tryptic digestion of the equivalent of 2.5 µg of C5 per ml and tumor ECF was 25 µl of the low molecular weight peak from the extract of each patient's tumor tissue.

¶ Percentage of enhancement of base-line random migration by 2.5 mM sodium ascorbate.

** Interval between initial symptoms and 24-h urine collection or assessment of in vitro response of patient's eosinophils.

tic large cell tumors of the three patients who lacked eosinophilia contained low molecular weight ECF in quantities of 0.04, 0.12 and 0.18 net eosinophils/hpf per mg in extracts of 0.6–3 g of tumor tissue. Low molecular weight ECF activity was absent from the pulmonary metastases of patient 5 and was less than 0.1 net eosinophil/hpf per mg in extracts of 2–4 g of three renal cell carcinomas and two colonic carcinomas from patients lacking eosinophilia.

Partial purification and characterization of tumor-derived low molecular weight ECF. The low molecular weight ECF activity from extracts of normal lung and tumor tissue and from concentrates of tumor cell culture media, urine and pleural fluid were applied to Dowex-1 in 0.1 M pyridine and eluted with a linear pyridine-formic acid gradient. A broad peak of ECF activity was detected in the pH range of 5.0 to 3.5 for extracts from tumors of patients 2 and 3 (Fig. 3) and for cell culture supernates from the same tumors and urine from the same patients (not shown). As the low molecular weight chemotactic factors derived from lung squamous cell

carcinomas clearly differed in charge from the ECF-A tetrapeptides of comparable size and eosinophil specificity, the tumor derived material was designated ECF-LSC (eosinophil chemotactic factor of lung squamous cell carcinoma). In contrast, the low molecular weight peak of ECF activity in the pleural fluid from patient 5 and in an extract of a nontumorous human lung surgical specimen obtained from a patient with bronchiectasis (normal lung extract) appeared in the more acidic range of pH 3.2 to 2.2, characteristic of the elution position of the preformed ECF-A tetrapeptides (6). Pools of low molecular weight ECF from Sephadex G-25 filtration of 1–5 g of tumor from patient 4 and 0.6–3 g each of the three anaplastic large cell lung tumors unassociated with eosinophilia, and the five nonlung tumors did not contain detectable activity after Dowex-1 chromatography.

High voltage paper electrophoresis (Fig. 4) of portions of the Sephadex G-25 low molecular weight peak of chemotactic activity in tumor cell culture supernates of patients 2 and 3 revealed a major peak of

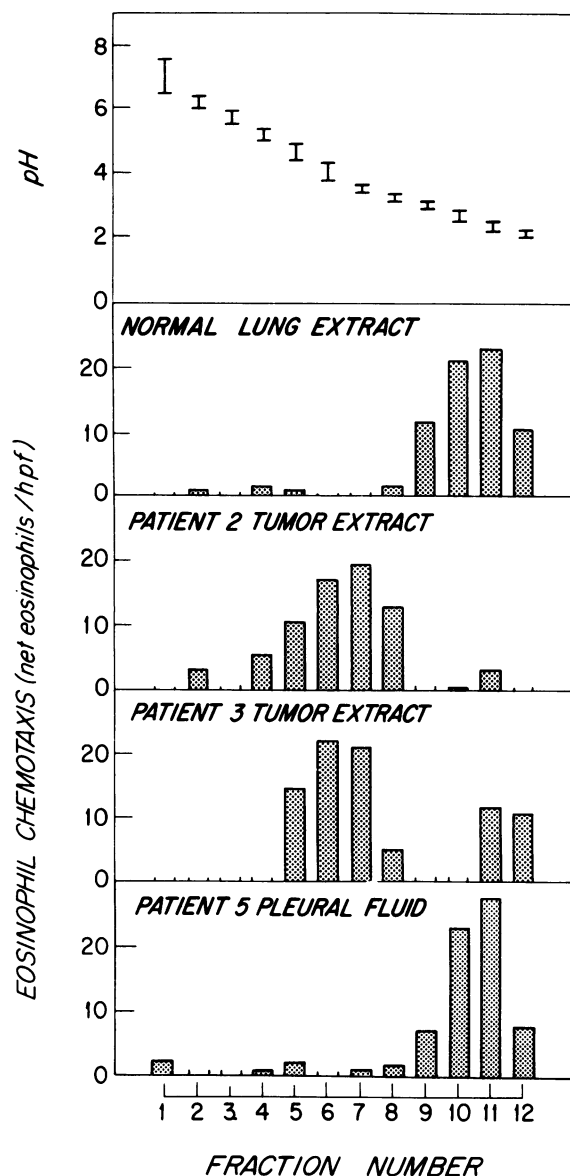


FIGURE 3 Dowex-1 fractionation of low molecular weight ECF in human lung tissue, tumor tissue, and pleural fluid. Pools of low molecular weight ECF from Sephadex G-25 column filtration of 1 g of tumor, 5 ml of pleural fluid (patient 5), and 5 g of nontumorous lung tissue fragments were lyophilized, redissolved in 0.1 M pyridine, and applied to Dowex-1 columns of 30-ml bed volume. The fractions eluted in the 0.1-M pyridine-0.5-M formic acid gradient were pooled as indicated by the pH range (vertical brackets) depicted in the upper panel, lyophilized, and redissolved in 2 ml distilled water. 0.1 ml of each fraction was employed as the stimulus in eosinophil chemotactic assays with cells from a patient with 71% peripheral blood eosinophilia.

eosinophil chemotactic activity that migrated anodally at pH 5.6 at approximately half the rate of the ECF-A tetrapeptide, Val-Gly-Ser-Glu, which is consistent with the less acidic nature of ECF-LSC. The low molecular

weight ECF activity from patient 3 derived from both tissue extracts (Fig. 3) and cell culture supernate (Fig. 4) contained not only ECF-LSC, but also additional components exhibiting a degree of acidity comparable to the ECF-A tetrapeptides and possibly even more acid material found between 32 and 38 cm (Fig. 4).

Portions of the pooled Dowex-1 peaks of low molecular weight ECF were incubated for 6 hr at 37°C with 10 μ g of subtilisin or trypsin per four maximal chemotactic doses of each principle. Subtilisin digestion inactivated the Dowex-1 pool of ECF-LSC from patient 2 tumor extract by 86% and from patient 3 tumor extract by 79% and inactivated the more acidic ECF from patient 5 pleural fluid by 83%; similar treatment with trypsin gave less than 20% inactivation of any of the samples. Subtilisin had previously been shown to inactivate the low molecular weight ECF of normal lung and of the tumor of patient 1 (5, 6). Thus both ECF-LSC from patients 2 and 3 and the more acidic ECF of patient 5 were comparable to the ECF-A tetrapeptides in terms of their susceptibility to proteolytic inactivation.

The leukocyte chemotactic preference of ECF-LSC at various stages of purification was examined in parallel with previously defined chemotactic stimuli by employing a range of concentrations of each factor and purified populations of target leukocytes. The chemotactic activities for neutrophils, eosinophils and monocytes are compared in Table III utilizing that concentration of each stimulus which achieved a neutrophil response of 15–25 net cells/hpf. The ratio of eosinophil to neutrophil chemotactic activity for ECF-LSC in the initial low molecular weight pool from Sephadex G-25 was 1.5 and increased to nearly three after Dowex-1 chromatography and high voltage electrophoresis on paper. The degree of eosinophil preference exhibited by ECF-LSC was similar to that for both the valyl tetrapeptide of ECF-A and the lipid factor HETE (Table III). None of these factors had appreciable chemotactic activity for mononuclear leukocytes. In contrast, C5 fragment was about equally chemotactic for eosinophils, monocytes, and neutrophils while kallikrein was preferentially chemotactic for neutrophils.

In vitro assessment of the capacity of the eosinophils of the patients to respond to C5 fragment and the low molecular weight ECF of their own tumors revealed a markedly depressed response for patient 2 and the loss of responsiveness for patient 3. These patients exhibited high levels of ECF-LSC in extracts of tumor and urine. In patient 3, simultaneous studies of his own eosinophils and low molecular weight ECF suggested a relationship of the decreased chemotactic responsiveness to high levels of urinary excretion of ECF-LSC. The eosinophil defect in patient 2 was profound and included not only chemotaxis, but also a poor response to sodium ascorbate which enhances random migration

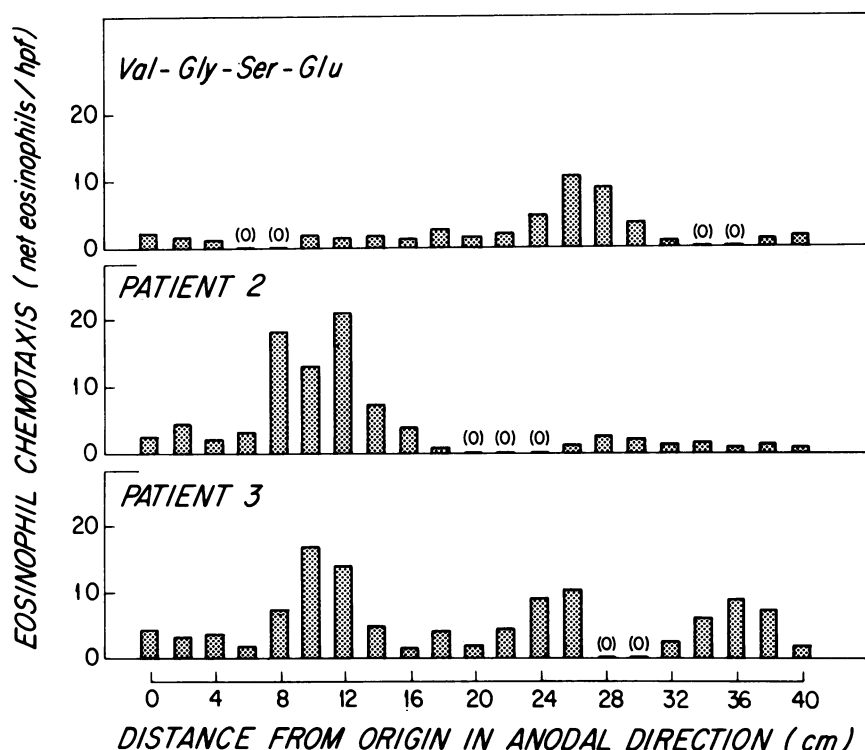


FIGURE 4 High voltage paper electrophoresis of low molecular weight ECF in tumor cell culture supernatant media. The Sephadex G-25 low molecular weight peak of eosinophil chemotactic activity from the equivalent of 5 ml of pooled culture medium of patients 2 and 3, and 20 nmol of purified synthetic Val-Gly-Ser-Glu were subjected to electrophoresis. 1/10 of the eluate from each 1-cm wide paper strip was assessed in duplicate for chemotactic activity employing eosinophils from a donor with 32% peripheral blood eosinophilia.

TABLE III
Preferential Chemotactic Activity of ECF-LSC

Cell type*	Kallikrein†	C5 fragment§	HETE ^a	ECF-A valyl-tetrapeptide¶	Tumor extract Sephadex G-25 low molecular weight peak (ECF-LSC)**	ECF-LSC Dowex-11‡	ECF-LSC paper electrophoresis§§
Neutrophils*	24.8±8.1	25.4±3.9	24.6±6.2	14.6±5.8	18.8±4.2	17.0±2.7	14.8±5.9
Eosinophils*	10.2±2.3	28.0±6.9	43.7±10.5	26.2±11.3	27.7±4.4	33.7±7.5	40.7±7.8
Mononuclear leukocytes*	13.6±5.8	21.4±4.4	6.6±3.8	3.5±2.1	8.1±4.0	4.1±3.3	5.2±3.7

* The purified leukocyte suspensions contained: neutrophils, 95–98% neutrophils and 2–5% mononuclear leukocytes; eosinophils, 42–84% eosinophils, 5–23% neutrophils, and 11–34% mononuclear leukocytes; mononuclear leukocytes, 69–77% lymphocytes, 18–24% monocytes, and 3–6% neutrophils. Results shown are mean±1 SD net leukocytes/hpf for three preparations of each cell type from separate donors utilizing the same source of each stimulus. Background counts in the absence of stimuli averaged: neutrophils, 4; eosinophils, 9; mononuclear leukocytes, 3.

† Kallikrein from human plasma (18) yielded 1.1 µg bradykinin/ml from 0.2 ml of heat-inactivated plasma.

§ C5 fragment was derived from trypsin treatment of 2.5 µg equivalent of C5/ml.

^a HETE concentration was 10 µg/ml (19).

¶ Val-Gly-Ser-Glu concentration was 0.1 µM.

** 1/10 of the pool of low molecular weight ECF from tumor extract of patient 2 (Fig. 2) was employed as the stimulus in each chemotactic chamber.

‡‡ 1/10 of a pool of fractions 6 and 7 for patient 3 (Fig. 3) was the stimulus.

§§ 1/10 of a pool of eluates from paper strips 8–12 cm from the origin for patient 2 (Fig. 4) was the stimulus.

of normal eosinophils by greater than 100% (15, 23). The Dowex-1 fraction of ECF-LSC obtained from an extract of tumor from patient 3 was compared with the more acidic fraction from normal lung and with synthetic ECF-A tetrapeptide for their capacity to deactivate eosinophils in vitro (Fig. 5). The concentrations of chemotactic factors were adjusted to those eliciting comparable eosinophil chemotactic responses. The Dowex-1 fraction from the tumor extract and from normal lung gave 40–50% deactivation by 5 min and 80% deactivation by 20 min; the valyl-tetrapeptide produced 80% deactivation by 5 min and complete chemotactic unresponsiveness by 10 min.

DISCUSSION

Anaplastic squamous cell lung carcinomas from three patients with profound bone marrow, peripheral blood, and tumor eosinophilia contained a low molecular weight factor chemotactic for human PMN leukocytes, with a preference for eosinophils (Tables I and II). This factor could be distinguished on the basis of charge from the ECF-A tetrapeptides previously recognized as constituents of normal human lung tissue (6, 24–26). The low molecular weight eosinophilotactic activity was lacking in extracts of an adenocarcinoma of the lung of a patient with mild peripheral blood eosinophilia, but without eosinophil infiltration of the tumor, a renal cell carcinoma metastatic to the lung which elicited a local eosinophilia only in the pleural space (Tables I and II), and numerous control tumors unassociated with

eosinophilia including three large cell anaplastic lung carcinomas, three renal cell tumors, and two colonic carcinomas. Furthermore, monolayer cultures of dispersed tumor cells from two of the patients with squamous cell lung carcinoma secreted low molecular weight eosinophilotactic activity. Extracts from each of the five tumors from the patients with eosinophilia also demonstrated high molecular weight neutrophil chemotactic activities and intermediate molecular weight chemotactic activities which were capable of attracting eosinophils and neutrophils (Fig. 1). These chemotactic activities were also present in the supernatant media of tumor cell cultures assayed over a period of approximately 10 mo (Fig. 2, Table II). Neither the intermediate molecular weight activities for eosinophils and neutrophils nor the high molecular weight neutrophil activities from the tumor extracts or the culture supernates were further characterized because factors of comparable size extracted from normal human lung or purified rat mast cells have not been sufficiently defined to permit a comparison on a basis other than size (27). In contrast, the peptide components of ECF-A have been structurally defined as being composed of at least two acidic tetrapeptides (6), thereby affording an opportunity to compare the physical and functional characteristics of the low molecular weight material elaborated by the squamous cell lung carcinomas with these acidic tetrapeptides.

The initial suggestion that tumor-derived low molecular weight ECF did not come from mast cells was based on the relative lack of tumor tissue histamine as compared to the quantity of eosinophil chemotactic activity (5). This point is now substantiated by the finding that the concentration of low molecular weight ECF in extracts of the squamous cell carcinomas was 2 to 35 times that of normal lung tissue while the histamine content of the tumor tissues was only 1/5 to less than 1/100 that of nontumor lung tissue (Table II). More critical was the long-term elaboration by dispersed tumor cell cultures of an eosinophil chemotactic peptide indistinguishable from the activity in the original tumor extracts by size (Fig. 2), charge (Fig. 4), and susceptibility to inactivation by subtilisin. The low molecular weight ECF activity extracted from the squamous cell lung carcinomas eluted from Dowex-1 in a peak in the less acidic region at pH 5.0–3.5, compared to the low molecular weight material extracted from normal human lung and from the pleural fluid of patient 5, which eluted at pH 3.2–2.2 in the same position as the ECF-A tetrapeptides (6, 27; Fig. 3). The migration in high voltage paper electrophoresis of the low molecular weight ECF activity from the squamous cell carcinomas again confirmed that it is less anionic than the tetrapeptides of ECF-A, although in the case of patient 3, but not patient 2, there were substantial quantities of more acidic low molecular weight ECF

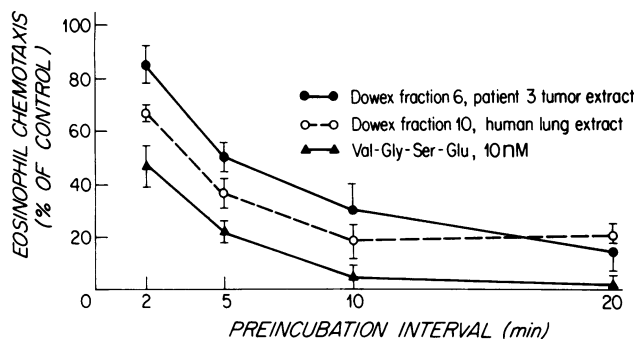


FIGURE 5 Eosinophil chemotactic deactivation by partially purified low molecular weight ECF from tumor and normal lung and by tetrapeptide. Unpurified eosinophils, from a patient exhibiting 53% eosinophilia, were preincubated for varying time periods with factors diluted to achieve chemotactic activities of 16 net eosinophils/hpf for Dowex-1 fraction 6 of patient 3 (Fig. 3), 14 for Dowex-1 fraction 10 of lung tissue (Fig. 3), and 10 for 10nM valyl-tetrapeptide. Eosinophils were then washed and placed in chambers containing a twofold higher level of Dowex-1 fraction 6 of patient 3 as the stimulus. This stimulus attracted 24 net untreated eosinophils/hpf and this response was set at 100%. Data plotted are the mean points and range of individual values (brackets) for two separate experiments with the same donor.

activities as well (Figs. 3 and 4). The low molecular weight ECF activity present in large amounts in the squamous cell lung carcinoma and elaborated by cultures of dispersed tumor cells is designated eosinophil chemotactic factor of lung squamous cell carcinoma (ECF-LSC) to distinguish it from the more acidic tetrapeptides derived from mast cells, where the activities appear to be associated with the metachromatic granules (26, 27). The designation ECF-LSC is arbitrarily descriptive so as to denote the source of this newly recognized ECF, but does not eliminate the possibility of other derivations.

ECF-LSC attracted eosinophils and neutrophils in a dose-response fashion. The preference of ECF-LSC for eosinophils compared to neutrophils and the relative lack of effect for monocytes was observed at each of the stages of purification. The degree of preference for eosinophils was equal to or greater than that of the synthetic tetrapeptide, Val-Gly-Ser-Glu, and the highly purified lipid HETE in the same three experiments (Table III). The eosinophil response required a concentration gradient indicating that ECF-LSC is a chemotactic factor. Furthermore, as is characteristic of other chemotactic factors including the ECF-A tetrapeptides (6, 14–16), pretreatment of eosinophils with ECF-LSC depressed their subsequent response to the homologous stimulus (Fig. 5), a phenomenon termed functional deactivation. The markedly depressed response of the eosinophils of patient 2 to C5 fragment or autologous ECF-LSC and the eventual loss of the chemotactic response of eosinophils from patient 3 are tentatively attributed to in vivo chemotactic deactivation.

Patients 2 and 3 had heterogenous polymorphonuclear leukocyte chemotactic activity in the urine, and Sephadex G-25 filtration of urine concentrates showed a prominent low molecular weight peak which was comparable in size and charge to ECF-LSC and also exhibited preferential activity for eosinophils (Fig. 2, Table II). ECF-LSC increased in the urine of both patients with progression of tumor growth, but was never detected in plasma or serum. The quantity of ECF-LSC excreted per 24 h reached a level equivalent to that extractable from 0.5 to 2.0 g of tumor tissue. Such excretion rates are compatible either with localized tumor necrosis or elaboration by viable tissue in a manner comparable to that of cultured cells. Although multiple chemotactic factors of differing leukocyte specificities were elaborated by the tumors, the small size of ECF-LSC may have facilitated access of high concentrations to eosinophils in the peripheral blood and possibly in the bone marrow stores, as manifested by the presence or development of apparent in vivo chemotactic deactivation. Thus ECF-LSC or a separate tumor-derived eosinopoietic factor or both acting in concert could have stimulated production of eosinophils and their release

into the circulation. The previously recognized capacity of malignant lung tumors to produce peptides normally secreted by other organs (28–32), suggests that ECF-LSC may be present in undetectable amounts in certain as yet undetermined normal tissues.

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