Digestion of the Fifth Component of Complement by Leukocyte Enzymes

Sequential Generation of Chemotactic Activities for Leukocytes and for Tumor Cells

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Leukocytes contain within their lysosomal granules enzymatic activity that will generate from C5 chemotactic activity for leukocytes (neutrophils) and tumor (Walker carcinosarcoma) cells. Similar activity has been found in phagocytic supernatant fluids from neutrophils and in purified preparations of the leukocyte neutral proteases elastase and cathepsin G. While leukotactic activities can be generated from either the third (C3) or the fifth (C5) components of complement, only C5 serves as a source for generation of the chemotactic activity for tumor cells. As has been previously shown with trypsin, the C5-related chemotactic activities generated by leukocyte proteases are time-dependent: leukotactic activity appears early, then disappears, and is replaced by chemotactic activity for tumor cells. The generation of these chemotactic activities from C5 is blocked by prior treatment of leukcoyte preparations with the neutral protease inhibitor Trasylol. The demonstration that enzyme activities from leukocytes have the ability to generate tumor cell chemotactic factors from C5 suggests a possible mechanism by which the development of metastatic lesions may be promoted at sites of tissue injury or inflammation. (Am J Pathol 94:75–84, 1979)

THE PASSAGE of circulating tumor cells into extravascular sites is an important step in the process of tumor metastasis. Like leukocytes, some neoplastic cells are capable of active, directed migration *in vitro* toward chemical stimuli.[1-3] In addition, there is experimental evidence that such chemotactic movement of tumor cells may be important *in vivo*. In the studies of Ozaki et al,[4] the injection of chemotactic factors for tumor cells into tissue sites resulted in the formation of metastases of circulating tumor cells at the sites of injection. Metastases did not occur at sites injected with vasopermeability factors or chemotactic factors for leukocytes.[4]

In vitro chemotactic activity for tumor cells can be generated from

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serum, from zymosan-activated serum, or from the fifth component of complement (C5). Crude extracts of normal tissue or tumor tissue [5] interact with normal serum or purified C5 to generate chemotactic activity for tumor cells.

Trypsin treatment of C5 also generates similar activity. During trypsinization of purified C5, chemotactic activity for leukocytes is first generated but, as digestion is continued, the chemotactic activity for leukocytes declines and is replaced by chemotactic activity for tumor cells.[2] Likewise, trypsin treatment of fractions from zymosan-activated serum generates chemotactic activity for tumor cells while abolishing chemotactic activity for leukocytes.[3]

In addition to trypsin, previous work has shown that leukocyte enzymes are also capable of generating chemotactic activity for leukocytes by the activation of complement and by the direct cleavage of C5.[6,7] Active enzymes in inflammatory exudates,[8] released during phagocytosis [7] and responsible for this activity, have been identified as the neutral proteases elastase and cathepsin G.[9] We sought to determine, therefore, if these same leukocyte enzymes were also capable of generating peptides from C5 with chemotactic activity for tumor cells. If it could be shown that leukocyte enzymes generate chemotactic peptides for tumor cells, this might relate to the observation that metastases occur with increased frequency at the site of tissue injury in experimental models.[10-12]

Materials and Methods

Ascites Tumor Cells

The Walker carcinosarcoma, maintained as an ascites tumor by serial passage, is the same tumor line used previously.[2,3] For study of chemotaxis, ascites fluids were harvested in Medium 199, treated by 0.83% ammonium chloride to lyse erythrocytes, washed three times in fresh Medium 199, and suspended at a concentration of 4×10^6 cells/ml in Medium 199 containing 10% fetal calf serum.

Neutrophilic Leukocytes (PMN)

Peritoneal exudates were induced in rabbits by the method of Cohn and Morse [13] and prepared for assay of chemotaxis as described before.[2,3]

Preparation of Complement Components

The purified third (C3) and fifth (C5) components of complement were prepared from normal human serum according to the method of Nilson et al.[14] The purity of the materials obtained was tested by immunodiffusion using goat antihuman C3, goat antihuman C5, and goat antinormal human serum. Each protein preparation formed a single precipitation band with its corresponding antibody and with antinormal human serum. C5 preparations did not form precipitation bands with anti-C3 nor did C3 preparations form precipitation bands with anti-C5. The homogeneity of the preparations was also confirmed by elec-

trophoresis in 4% polyacrylamide gels (Biorad Laboratories, Richmond, Calif.) in the presence of 0.1% SDS buffer.[14] Each preparation was concentrated, dialyzed against phosphate-buffered saline, and stored at −70 C until use. The concentration of the protein in each sample was determined by the method of Lowry et al.[15]

Leukocyte Enzyme Preparations

The preparation and quantitation of enzymatic activities in phagocytic supernatant fluids, leukocyte granule extracts, and purified enzyme preparations are described in detail in a recent publication.[9] The enzymatic activities of the purified enzyme preparations were quantitated using purified elastase and cathepsin G as standards. The enzymatic activities of phagocytic supernatant fluids and crude granule extracts were normalized to a final concentration of 1% elastase activity (w/w).

Conditions for Digestion of Complement Components

For each enzyme or leukocyte preparation to be tested, 250 μg of purified C5 was incubated with 1% (w/w) purified enzyme or 1% (w/w) elastase equivalents of phagocytic supernatant fluids or granule extract.

Timed digestions were conducted at 37 C (pH 7.2 to 7.4) in volumes of 2 ml. At the end of each period, the digestion was stopped by the addition of 4% (w/w, final concentration) soybean trypsin inhibitor and the volume of the reaction mixture was brought to 5 ml by the addition of Medium 199 without fetal calf serum. Controls were prepared by incubating C5, enzyme preparations, and buffer solutions individually using identical conditions to those described. Digestion of C3 was conducted in parallel experiments with the exception that higher concentrations of C3 were required to detect chemotactic activity for leukocytes (1250 μg of C3 was digested with 1% enzyme [w/w] or leukocyte enzyme preparation). The same controls were employed as in experiments in which C5 was studied.

Enzyme Inhibition and Antibody Blocking

Trasylol (Bayer AG, Wuppertal, West Germany) was bound to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NY) by cyanogen-bromide linkage as described by Baugh and Travis.[16] In certain experiments the enzyme preparations were passed over a column containing ~ 2 mg of the bound enzyme inhibitor prior to incubation with C5 or C3. The purpose of using the enzyme inhibitor in immobilized form was to avoid contamination of the migrating cells by this agent.

Antibodies to C5 were raised in goats and were characterized as described previously.[8] In experiments designed to test the suppressive effect of antibody on preformed chemotactic activity, 10-μl and 25-μl volumes of goat serum containing anti-C5 were incubated at 37 C for 30 minutes with reaction mixtures containing 50 μg of C5 which had been enzymatically digested. Controls included treatment of reaction mixtures with identical volumes of normal goat serum. The inhibition of chemotactic activity caused by these treatments was expressed in terms of percent reduction of chemotactic movement based on values obtained in untreated samples and negative controls.

Assays of Cell Migration and Chemotaxis

The method of Romualdez and Ward[8] was modified as follows: the micropore filters used for studies of tumor cell migration were composed of nitrocellulose with a porosity of 12 μ (Selectron Filters, Schleicher and Schnell, Keene, NH). Each assay of leukocyte chemotaxis was performed in duplicate, and the numbers of cells migrating in two high-power fields were read on each filter. Each assay of tumor cell chemotaxis was performed in triplicate, and the number of cells migrating in three high-power fields was determined in each filter. The mean and standard error were calculated.

Results

Generation by Leukocyte Enzymes of Chemotactic Activity for Leukocytes and Tumor Cells

The supernatant fluids from suspensions of human neutrophils that had ingested opsonized zymosan particles generated chemotactic activity for leukocytes and for tumor cells when incubated with C5 (Table 1). The generation of leukotactic activity occurred shortly after the onset of incubation; maximum chemotactic activity for leukocytes was generated within 2 to 5 minutes. Chemotactic activity for tumor cells appeared later than leukotactic activity (~ 60 minutes), and its appearance was accompanied by a fall in leukotactic activity. The sequential appearance of the two chemotactic activities after different periods of incubation was also observed when trypsin was used as a generator.

Neither leukocyte-directed nor tumor-cell–directed chemotactic activity was observed in samples of C5 incubated in the absence of a proteolytic enzyme or in preparations of enzymes incubated in the absence of C5.

Leukotactic activity was also generated from C3 after 2 minutes of similar treatment. However, quantities of this substrate required to generate an amount of chemotactic activity comparable to that from 50 μg of C5 were 5-fold larger, ie, 250 μg of C3. Even when this quantity of C3 was employed in each reaction, the generation of chemotactic activity for tumor cells was not observed.

We next looked at the ability of crude granule lysate homogenates and purified granule enzymes to generate chemotactic activities from C3 and C5.

The results of treatment of C5 by the purified lysosomal enzymes

Table 1—Generation of Chemotactic Activity for Leukocytes and for Tumor Cells by Treatment of C3 and C5 With Leukocyte Enzymes

	Number of cells/high-power field ± SEM			
	2-minute digestion		60-minute digestion	
Chemotactic factor	PMN	Tumor	PMN	Tumor
C3* alone	21 ± 8	12 ± 2	5 ± 2	13 ± 1
C3* + phagocytic supernatant	98 ± 8	12 ± 2	43 ± 8	18 ± 2
C3* + trypsin	213 ± 3	17 ± 2	94 ± 8	16 ± 2
C5† alone	27 ± 6	11 ± 1	24 ± 5	16 ± 3
C5† + phagocytic supernatant	>300	13 ± 1	19 ± 3	40 ± 4
C5† + trypsin	130 ± 31	4 ± 2	20 ± 10	45 ± 8

* 250 μg C3 per chemotaxis chamber
† 50 μg C5 per chemotaxis chamber

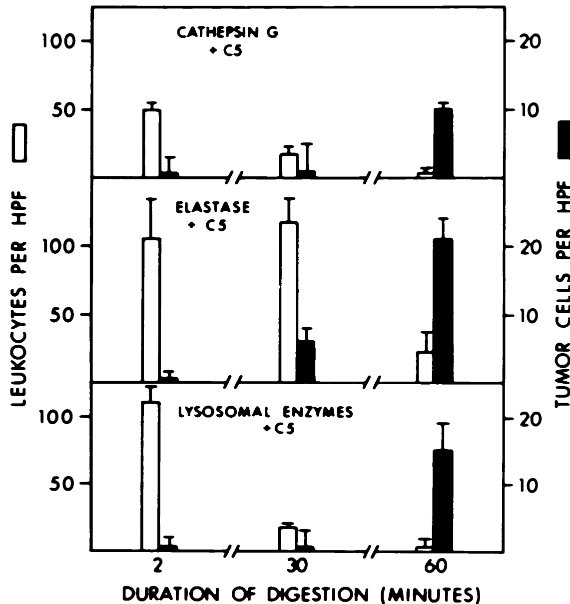
cathepsin G and elastase, as well as with leukocyte granule extracts, were similar. In each case, there was initial generation of leukotactic activity followed by a decline in this activity with time and by the generation of chemotactic activity for tumor cells (Text-figure 1). The treatment of C3 with these preparations generated chemotactic activity for leukocytes but not for tumor cells (Text-figure 2).

Inhibition of the Generation of Chemotactic Activities by Affinity Removal of Enzyme Preparations With Trasylol

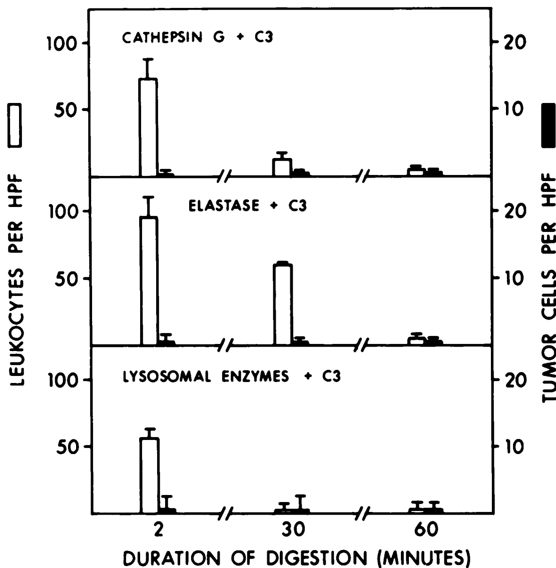
To study the nature of the interaction between the leukocyte enzymes and C5, the enzyme preparations were pretreated by passage through Trasylol columns prior to incubation with C5 (Table 2). Compared with nontreated enzymes, the treated preparations (passed through Trasylol-containing columns) lacked virtually all ability to generate either leukocyte-directed or tumor-cell–directed chemotactic activity. Controls (Medium 199 to which no enzyme had been added) were also passed through the Trasylol columns; this treatment had no inhibitory effect on the random movement of leukocytes or tumor cells.

Inhibition by Treatment With Antiserums of the Chemotactic Activity Generated From C5

To confirm that the chemotactic activities for tumor cells and leukocytes were derived from the fifth component of complement, and not from



TEXT-FIGURE 1—Generation of chemotactic activity for leukocytes and tumor cells from C5 by digestion with enzymes. 50 μg C5 (sufficient for one Boyden chamber assay) was digested with 1% enzyme (w/w) (see *Materials and Methods*) at 37 C for the stated times. The reaction was then stopped by the addition of 4% (w/w) soybean trypsin inhibitor. Chemotactic activity expressed represents the actual number of cells migrated per high-power field minus values obtained for migration induced by C5 alone.



TEXT-FIGURE 2—Generation of chemotactic activity for leukocytes and tumor cells from C3 by digestion with leukocyte enzymes. 250 μg C3 (sufficient for one Boyden chamber assay) was digested with 1% enzyme (w/w) (see *Materials and Methods*) at 37 C for the stated times. The reaction was then stopped by the addition of 4% (w/w) soybean trypsin inhibitor. Chemotactic activity expressed represents the actual number of cells migrated per high-power field minus values obtained for migration induced by C5 alone.

a contaminant, the effect of anti-C5 on the generated chemotactic activity was analyzed. Following periods of enzymatic digestion appropriate to the generation of chemotactic activity for leukocytes (2 minutes) and tumor cells (60 minutes), the preparations were incubated with serum containing antibody to C5. Inhibition of both leukocyte and tumor cell chemotactic activities was accomplished using serums containing anti-C5 but not by using goat serum (Table 3).

Discussion

A major step in the establishment of distant metastases is the movement of circulating neoplastic cells from lymphatic channels or blood vessels into extravascular tissues. Potential, nonexclusive mechanisms for such transvascular passage include the direct invasion and destruction of the endothelium and basement membrane by tumor cells [17] and the active migration of cells across the vessel wall.

There is growing evidence that tumor cells are capable of active directed migration into extravascular tissues. Electron microscopic studies of developing metastases have demonstrated extension through intercellular junctions between endothelial cells of the pseudopods of intravascular tumor cells.[18,19] This is similar to the passage of leukocytes across vessels in inflammatory sites.[20] Both leukocytes and tumor cells are capable of chemotactic responses *in vitro*.[3,21] The studies of Ozaki et al indicate that *in vivo* the injection of chemotactic factors for tumor cells

Table 2—Inhibition of the Generation of Chemotactic Activity From C5 by Pretreatment of Enzymes With Trasylol

	Number of cells/high-power field ± SEM					
	PMN			Tumor		
Preparation incubated with C5*	Enzyme without treatment	Trasylol-treated enzyme	Percent inhibition	Enzyme without treatment	Trasylol-treated enzyme	Percent inhibition†
Phagocytic supernatant	116 ± 6	40 ± 4	100	41 ± 3	10 ± 1	100
Cathepsin G	119 ± 12	55 ± 17	100	40 ± 5	15 ± 2	100
Elastase	93 ± 5	35 ± 10	100	46 ± 7	17 ± 4	97
Lysosomal enzymes	183 ± 12	66 ± 16	91	37 ± 3	17 ± 3	95

* Migration of PMN and tumor cells toward untreated C5: PMN = 55 ± 9 cells/high-power field; tumor cells = 16 ± 3 cells/high-power field.
† Percent inhibition of chemotactic activity

into tissue sites will result in the formation of metastases of circulating tumor cells at the sites of injection but not at sites injected with vasopermeability factors or substances chemotactic for leukocytes.[4]

Our experiments demonstrate the existence of a potential mechanism for the generation *in vivo* of factors which have chemotactic activity for neoplastic cells. Enzymes, located within the lysosomal granules of human leukocytes or within other tissues,[5] are able to act on C5 to generate leukotactic factors or chemotactic activity for tumor cells, depending on the conditions of incubation. The interaction between C5 and leukocyte enzymes could occur readily in injured tissues or at sites of inflammatory responses since it has been shown that leukocytic lysosomal enzymes, capable of activating C5, are released during phagocytosis[7] and are present in inflammatory effusions.[8] There is experimental evidence that the metastasis of circulating tumor cells is more frequent at sites of trauma than in normal tissues,[10-12] although the models used in these experiments

Table 3—Inhibition of Chemotactic Activity for Leukocytes and for Tumor Cells by Treatment With Anti-C5

	% Inhibition of chemotactic activity	
	PMN	Tumor
C5 + phagocytic supernatant alone	0*	0†
C5 + phagocytic supernatant + anti-C5‡	100	66
C5 + phagocytic supernatant + normal goat serum‡	0	8

* 250 ± 21 cells migrated per hpf. Negative control (C5 alone), 28 ± 15 cells migrated per hpf.
† 33 ± 4 cells migrated per hpf. Negative control (C5 alone), 7 ± 2 cells migrated per hpf.
‡ 25 μl of serum/50 μg of C5

do not exclude the possibility of passive emigration of tumor cells due to vascular trauma. Our experiments indicate that lysosomal granule enzymes released either during phagocytosis or isolated from lysosomal granules have the ability to generate chemotactic activity for tumor cells.

We are able to block the generation of the chemotactic activities for leukocytes or tumor cells by passage of phagocytic supernatants or lysosomal enzyme preparations over affinity columns of Trasylol–Sepharose. These columns separate elastase and chymotrypsin-like enzymes from other proteins in granule extracts, due to specific interactions of these enzymes with Trasylol.[16] Therefore, it is suggested that the principal neutral proteases in neutrophilic granules, ie, elastase and cathepsin G (the leukocyte chymotrypsin-like enzyme), account for the major tumor cell chemotactic factor generator activity in leukocyte phagocytic supernatants and homogenates of lysosomal granules. These observations are consistent with recently published data from this laboratory indicating that these enzymes also have the ability to inactivate C3-derived and C5-derived chemotactic factors for leukocytes.[9]

Chemotactic activity for leukocytes can be generated from purified C3 by appropriate treatment with trypsin [2,22,23] or plasmin.[24] However, in our experience, it has not been possible to generate chemotactic activity for tumor cells from C3 after any of a variety of conditions of trypsinization. In the experiments presented here, leukotactic activity was generated from C3 by leukocyte-derived enzymes, but only when the quantities of C3 used were five times greater than those amounts of C5 required to generate chemotactic activity for leukocytes or tumor cells. The observation that chemotactic activity for tumor cells can be generated from C5 but not from C3 is evidence of both the specificity of the C5-derived activity and the purity of the C3 substrate. Assuming that the leukotactic activity derived by trypsinization of C3 was due to contamination of the substrate by C5, we would have expected to observe the generation in these samples of chemotactic activity for tumor cells after 60 minutes of digestion. This is because leukocyte responses of the magnitude observed require from 35 to 50 μg C5 in our system. These amounts of C5, when trypsinized, generate detectable chemotactic activity for tumor cells. Thus, contamination of the C3 preparation with C5 can not explain the generation of leukotactic activity for neutrophils.

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