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Immunology

Chemotactic factor for tumor cells derived from the C5a fragment of complement component C5

(chemotaxis/leukocytes)

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ABSTRACT **Previously, we have established that the fifth component of complement (C5) serves as an important source of mediators that have locomotory (chemotactic) activity for leukocytes and tumor cells. C5a, a fragment (M_r 11,200) derived from the NH_2-terminal portion of the α chain of C5, is the major chemotactic peptide for leukocytes. The present studies demonstrate that cleavage of C5a with trypsin generates a derivative peptide that is chemotactic for tumor cells (Walker carcinosarcoma). This fragment has an estimated M_r of 6000 as assessed by gel filtration and does not require the COOH-terminal arginine of C5a, because equivalent amounts of chemotactic activity for tumor cells can be generated from des-Arg-C5a by digestion with trypsin. The C5a-derived chemotactic peptide for tumor cells demonstrates peak activity at approximately 1 pM. These studies emphasize the key role of the C5a region of the C5 molecule in the generation of peptides that affect locomotory responses of cells.**

Peptides originating from the complement system have potent biological activities related to locomotion of cells. In turn, these locomotory responses appear to be important in the biological behavior of both leukocytes and tumor cells. With leukocytes, the chemotactic peptides induce the emigration of cells from the vasculature, resulting in an inflammatory response (1). In the case of tumor cells, the biological outcome *in vivo* of the interaction of chemotactic peptides with tumor cells is not known, but it probably affects the behavior of the cells both locally (in the primary tumor) and at a distance (metastatic tumor cells). It is now known that the fifth component of complement (C5) is a major source of factors that cause unidirectional (chemotactic) migration of leukocytes towards a concentration gradient of attractant (2). Both leukocytes and malignant cells can respond to chemotactic factors derived from C5, although the factors to which they respond are not chemically identical (3, 4). There is considerable evidence that specific receptors for chemotactic factors exist on plasma membranes of leukocytes and that the interaction of a chemotactic factor with a neutrophil induces a change in membrane potential of the cell, with increased membrane permeability of Na^+, K^+, and Ca^{2+} and an increase in cytosol concentrations of free Ca^{2+} (reviewed in ref. 1). There is also some evidence that the chemotactic response of the leukocyte, like that of bacteria, requires methylation of a membrane protein, presumably involving the donor *S*-adenosylmethionine. All of the above phenomena precede the condensation of actin filaments in the leading edge of the cell, where the contractile event and, thus, cell locomotion, occurs (5).

The major chemotactic peptide from C5 is C5a, which induces leukocyte chemotaxis at 10 to 1 nM (2). This molecule, whose complete amino acid sequence has recently been established (6), consists of 74 amino acids (accounting for a M_r of 8200) and an oligosaccharide that is attached to residue 64 and accounts for an additional M_r of 3000. C5a has a COOH-terminal arginyl residue, which appears to be important for the muscle contracting activity (anaphylatoxin) of the molecule and augments the amount of chemotactic activity in the peptide (6).

Previously, we have established that the C5-derived tumor cell chemotactic peptide is distinct from the leukotactic peptide (4). Trypsin treatment of C5-derived leukotactic fragment(s) generates a molecule with tumor cell chemotactic activity that by gel filtration seems to have a smaller molecular weight than C5a (M_r 11,200) (6). This suggests derivation of the tumor cell chemotactic factor from the C5a molecule. In this report, we provide direct evidence that the tumor cell chemotactic factor is a derivative peptide of the C5a molecule and that this chemotactic factor for tumor cells behaves identically (functionally and in gel filtration) with the similarly active fragment generated by trypsin digestion of intact C5. The concentration of the peptide inducing a half-maximal chemotactic response for tumor cells (ED_{50}) is approximately 1 pM. These data stress the central role of C5 as a source for chemotactic peptides that affect locomotory responses of cells.

MATERIALS AND METHODS

Chemotaxis Assays. These assays were performed as outlined in earlier publications (3, 4). Modified Boyden chambers with micropore filters (Schleicher & Schuell) of 12-μm porosity were used. The upper compartment of each chamber was filled with 1.0 ml of tumor cells suspended at a concentration of 5×10^5/ml in 5% fetal calf serum. Hanks' medium was used for all suspensions and dilutions. The lower compartment of each chamber was filled with 1.0 ml of test material. Tumor cells were obtained by shaking of flasks that contained confluent and overgrown cultures of Walker carcinosarcoma cells maintained in culture continuously with Hanks' medium containing 5% fetal calf serum (4). All assays were done in triplicate and the chambers were incubated for 4 hr at 37°C in a humidified chamber containing air with 5% CO_2. For quantitation, migrated cells were counted by light microscopy in eight high-power fields (×150) selected at random. Data were expressed as mean counts of migrated cells ± standard error of the mean. Details of these procedures are fully provided in a recent publication (4). Chemotactic responses of the leukocytes were assessed by using rabbit peritoneal neutrophils (induced by glycogen). In this case, micropore filters of porosity 0.65-μm were employed and all cells migrating into channels of filters were counted in five light microscopy fields at ×150.

Enzyme Release Induced by Chemotactic Factor. Release of lysosomal enzymes from leukocytes pretreated with cyto-

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Abbreviation: C, complement.

chalasin B and then incubated with chemotactic factor is a convenient and rapid method to assess interaction of a chemotactic factor with leukocytes (7). In these experiments 1×10^7 leukocytes in 1.0 ml of Hanks' buffer are first treated with 10 μg of cytochalasin B (Aldrich). In triplicate 0.5 ml of the cell suspension was added to 0.1 ml of 1:3 serial dilutions of the test chemotactin. The reaction mixtures were incubated at 37°C for 5 min, and then the cells were removed by centrifugation. The supernatants were then assayed for lactate dehydrogenase (cytoplasmic marker) and glucosaminidase release. For the glucosaminidase assay, 400 μl of 4 μM *p*-nitrophenyl-*N*-acetyl-D-glucosamine was added to the cell supernatants and incubated for 30 min at 37°C. To stop the reaction, 0.5 ml of 0.4 M glycine buffer (pH 10.5) was added to the solution and the resulting color was read at 410 nm. All data are expressed as induced enzyme release, with the ED_{50} representing the amount of chemotactin required to give half-maximal enzyme release (7).

Preparation of C5a and Des-Arg-C5a Peptides. The chemotactic peptide C5a was generated in batch quantities by zymosan activation of fresh human serum in the presence of 1 M ε-aminocaproic acid. The peptide was then isolated by a combination of molecular-sieve and ion-exchange chromatographic techniques that are described in detail elsewhere (2). The final product stained as a single band in both analytical acidic and 1% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Des-Arg-C5a was produced by treatment of the purified C5a with carboxypeptidase B. For trypsin digestion of the C5a and des-Arg-C5a, peptides suspended in phosphate-buffered saline were treated with trypsin (1% wt/wt) for 30 min at 37°C, followed by addition of soybean trypsin inhibitor (Sigma), to give 2% (wt/wt). All enzyme preparations were purchased from Worthington.

Gel Filtration. To compare the chromatographic behavior of the tumor cell chemotactic factor generated from C5a with that generated from intact C5, trypsin-digested C5a was subjected to gel filtration on Sephadex G-75 superfine (Pharmacia). The gel was equilibrated in phosphate-buffered saline (pH 7.4) and poured into a 10×0.9 cm column. Flow rate was maintained under 20 cm H_2O of pressure. One to 3 μg of C5a or C5 trypsin-digested as above was applied to the column and 0.3-ml samples were collected. To monitor the protein at such low concentrations, fluorescamine (Fluram, Roche Laboratories) was used to increase the sensitivity of protein detection (8); 100 μl of each fraction was added to 1.4 ml of 50 mM sodium phosphate, pH 8.0. To each of these samples, 0.5 ml of fluorescamine solution (0.3 mg/ml in acetone) was added while the samples were being vigorously mixed. The samples were incubated at room temperature for 10 min and their fluorescence was read in a Perkin-Elmer/Hitachi fluorescence spectrophotometer with an excitation wavelength of 390 nm and the monitored emission wavelength of 475 nm.

RESULTS

Biological Activity of C5a and Des-Arg-C5a for Leukocytes. Dose–response curves of the two peptides for induced enzyme release (glucosaminidase) were determined, using peptide doses from 3.5 to 85 ng. The results with rabbit peritoneal neutrophils are seen in Fig. 1. C5a caused progressive enzyme release that could be seen first when 9 ng of C5a was used. At higher doses, a rapidly increasing amount of enzyme was released. By calculation of total enzyme content (determined by detergent-induced lysis of cells) the ED_{50} for C5a acting on rabbit peritoneal neutrophils was 35 ng. When human blood neutrophils were employed, the ED_{50} value was 4.5 ng. When the peptide des-Arg-C5a was used, little or no enzyme-releasing activity was demonstrated, although at the highest concentration (85 ng) a slight elevation above the base line value was observed (Fig. 1). The lack of enzyme-inducing activity for des-Arg-C5a was seen when either rabbit or human neutrophils were used. These data are in accord with those of a recent publication (2): C5a is a highly active chemotactic peptide, whereas des-Arg-C5a has markedly reduced chemotactic activity.

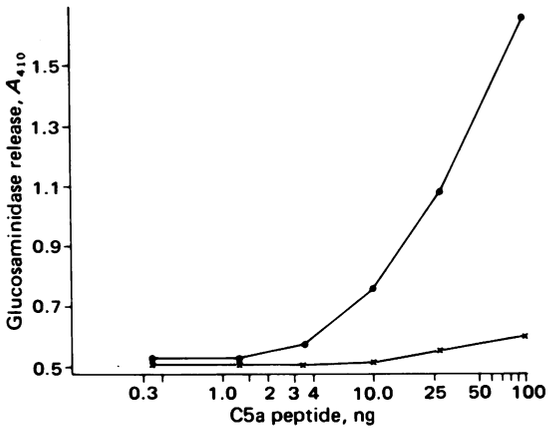

FIG. 1. Biological activity (lysosomal enzyme release) of C5a and des-Arg-C5a for leukocytes. Dose responses of these peptides incubated with rabbit peritoneal neutrophils reveal a rapidly rising cell response (release of glucosaminidase from cytochalasin B-treated leukocytes) caused by C5a (●), with an extrapolated ED_{50} of 34 ng. Des-Arg-C5a (×) has no significant biological activity ($ED_{50} \gg 83$ ng). When human blood neutrophils were used, the ED_{50} for C5a was 4.5 ng, whereas the ED_{50} for des-Arg-C5a was 83 ng.

Biological Activity of Trypsin-Digested C5a and Des-Arg-C5a. Small amounts (1 μg) of C5a and des-Arg-C5a were digested with trypsin and the dose response for chemotaxis of Walker tumor cells was determined. Neither intact C5a nor des-Arg-C5a had significant chemotactic activity for tumor cells (Fig. 2). The data in Fig. 2 reveal that, after trypsin digestion, both C5a and des-Arg-C5a contained chemotactic activity for Walker tumor cells. The dose–response curves were rather narrow, with activity first detectable when the amount of peptide treated with trypsin ranged from 0.5 to 20 ng. Maximal activity was found at approximately 5 ng, above which the cell responses rapidly diminished. Assuming complete proteolytic digestion of C5a and des-Arg-C5a with resultant 100% conversion to the tumor cell chemotactic factor, the des-Arg-C5a appears to be chemotactically active in the range of 1 pM, on

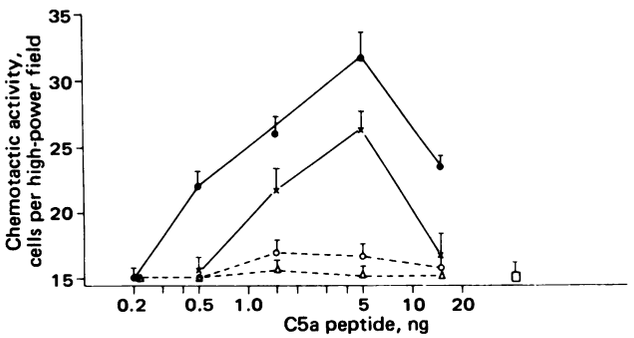

FIG. 2. Dose responses of Walker tumor cells with increasing concentrations of various peptides. Trypsin-digested C5a (●—●) and des-Arg-C5a (×—×) show chemotactic activity for tumor cells, with a maximum cell response at 5 ng/ml, approximately 1 pM. The C5a (○---○) and des-Arg-C5a (△---△) peptides not digested with trypsin reveal no such activity. □, Hanks' medium.

the basis of a molecular weight of approximately 11,200 for C5a and des-Arg-C5a (6). If this assumption is valid, the tumor cell factor appears to be severalfold more active than the leukotactic peptides with ED_{50} values in the range of 1000–10 pM (2, 6). This reduction in cell responsiveness at higher doses of the chemotactic factor is typically seen with both tumor cells and leukocytes and represents a cell "deactivation" phenomenon (9, 10).

Comparison of Leukotactic and Tumor Cell Chemotactic Activities in Gel Filtration. C5a and trypsin-digested C5a were chromatographed on Sephadex G-75 superfine in phosphate-buffered saline (pH 7.4) and the eluates were analyzed for leukotactic activity and tumor cell chemotactic activities. To improve the sensitivity for detection of protein, we have used the fluorescamine method of Böhlen *et al.* (8). Also to improve resolution, Sephadex G-75 superfine was used. In Fig. 3, 1 μg of intact C5a and 2 μg of trypsin-digested C5a were mixed prior to loading onto the column. The chromatographic patterns shown in Fig. 3 reveal the anticipated location of leukotactic-associated activity in the intact C5a preparation eluting near the position of the cytochrome *c* marker (M_r 12,000), whereas the tumor cell chemotactic activity eluted at considerably delayed position, slightly beyond the insulin marker, in fraction 28. The analysis of the trypsin-digested C5a preparation by fluorescamine-associated fluorescence revealed two peaks (Fig. 3*D*), one in the position of the C5a, which presumably represents residual C5a, and a second peak of fluorescence intensity, in fractions 27–29, that is associated with positions of tumor cell chemotactic activity. [It can be estimated that the tumor cell chemotactic factor has a M_r slightly less than that of insulin (6000).] The slight displacement of the fluorescence peak (Fig. 3*D*, fractions 27–29) with respect to the chemotactic activity for tumor cells (Fig. 3*C*, fractions 26–29) suggests that there may be some heterogeneity of peptide content in these fractions. In experiments not shown, non-trypsin-digested C5a preparation demonstrated only a single fluorescence (fluorescamine-induced) peak, which had the same center as the peak of leukotactic activity (Fig. 3*B*).

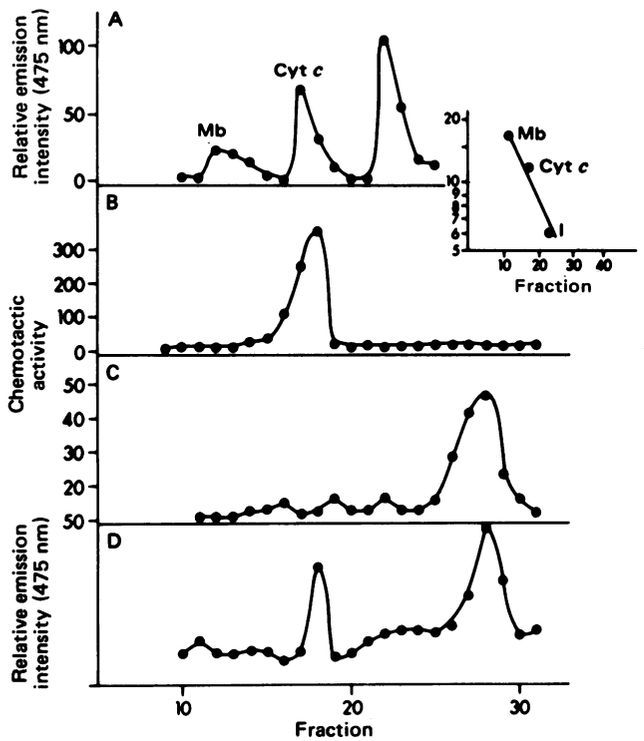

FIG. 3. Gel filtration in Sephadex G-75 (superfine) of C5a and trypsin-digested C5a. (*A*) Elution positions of reference markers myoglobin (Mb), cytochrome *c* (Cyt *c*), and insulin (I). (*Inset*) Molecular weight of reference markers, × 10^{-3}, vs. fraction number. (*B*) Elution position of neutrophil leukotactic activity in C5a, coinciding with the position of cytochrome *c*. (*C*) Late-eluting position for tumor cell chemotactic activity in the trypsin-digested C5a preparation. (*D*) Spectrophotometric results with fluorescamine-induced fluorescence, revealing some material coinciding with the C5a position, but a new peak of fluorescence associated with the area containing chemotactic activity for tumor cells.

DISCUSSION

The data presented in this paper provide evidence that the peptide with locomotory (chemotactic) activity for tumor cells is a derivative of C5a, the main chemotactic peptide for leukocytes. Thus, leukotactic as well as the tumor cell chemotactic activities and spasmogenic (anaphylatoxin) activities arise from the same portion of the C5 molecule, the NH_2-terminal portion of the α chain of C5 (2). The removal of the COOH-terminal arginyl residue (number 74) from C5a deletes the anaphylatoxin activity, the leukotactic activity, and, as shown here, the enzyme-releasing activity for neutrophils. The data in the present paper suggest that, insofar as the tumor cell chemotactic factor is concerned, the presence or absence of the COOH-terminal arginyl residue in the cleavage product of C5a is of little consequence for the expression of chemotactic activity for tumor cells. Furthermore, on the basis of the gel filtration characteristics, the tumor cell factor may be a fragment that is slightly less than half the size of the C5a peptide. Whether the chemotactic activity for tumor cells is associated with the COOH-terminal half, the NH_2-terminal half, or some other portion of the C5a peptide cannot be determined on the basis of available information.

That biological activity of the tumor cell chemotactic factor occurs at very low concentrations (1 pM) of the C5a-derived peptide indicates the extraordinary biological activity of this material. Indeed, compared with the leukotactic peptide (C5a), the derivative peptide (the tumor cell chemotactic factor) is more active by a factor of nearly 10^2.

There is evidence that most normal tissues contain a protease that, at neutral pH, will generate from C5 a chemotactic activity for tumor cells (11). The activity so generated is of low M_r (<30,000), is related immunochemically to C5, and is probably derived from the region of the C5 molecule from which C5a originates (4). Although there appears to be considerable structural diversity for the leukotactic factors in that other sources also exist (e.g., the third component of complement, bacteria, collagen, lymphocytes, casein), the factors that are chemotactic for tumor cells are far more limited in structure and include the fragment from C5 described in this report and a recently discovered product from resorbing bone (12). This latter material is not immunochemically related to C5. Thus, there are at least two different structural bases for chemotactic activity for tumor cells. At least with leukocytes, the diversity of chemotactic factors is related to the presence of different binding sites (receptors) on the cell surface. For instance, the synthetic leukotactic peptide *N*-formyl-Met-Leu-Phe binds specifically to receptors on the neutrophil surface. These receptors are different from the receptors for C5a (13, 14). Binding of chemotactic peptides occurs on the plasma membrane, and the binding is saturable, specific, and displaceable but is entirely resistant to influences of a structurally different chemotactic factor (15). It seems likely that tumor cells will also be found to have specific receptors for the chemotactic factors and that these receptors will be different from those receptors for C5a on the leukocyte.

The data in this report emphasize that two chemotactic factors with entirely different specificities are derived from the same general region of the C5 molecule. It is possible that the portions of each peptide responsible for triggering events leading to chemotactic migration may be similar or identical, although the requirement for the COOH-terminal arginine residue of C5a for leukotactic activity and its nonessentiality for tumor cell chemotactic activity suggests that, if the regions in the two peptides are overlapping or structurally related, they are not identical. Only the isolation and the structural definition of the tumor cell chemotactic peptide from C5a will provide answers to these questions.

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