Identification of the C5a des Arg Cochemotaxin
Homology with Vitamin D-binding Protein (Group-specific Component Globulin)

H. Daniel Perez, Edward Kelly, Dennis Chenoweth, and Fred Elfman
Rosalind Russell Arthritis Research Laboratory, University of California, San Francisco, San Francisco, California 94143; The Medical Service, San Francisco General Hospital, San Francisco, California 94110; and Travenol Laboratories, Roundlake, Illinois 60073

Abstract

The chemotactic activity of human C5a des Arg is enhanced significantly by an anionic polypeptide (cochemotaxin) in normal human serum and plasma. The cochemotaxin attaches to sialic acid residues within the oligosaccharide chain of native C5a des Arg to form a complex with potent chemotactic activity for human PMN. We investigated the nature of the cochemotaxin and found that vitamin D-binding protein is the putative cochemotaxin. Vitamin D-binding protein enhanced the chemotactic activity of native C5a des Arg, but had no effect on the chemotactic activity of either native C5a or FMLP. Sialic acid prevented both enhancement by vitamin D-binding protein of the chemotactic activity of native C5a des Arg and formation of C5a des Arg–vitamin D-binding protein complexes, detected by molecular sieve chromatography. Furthermore, vitamin D-binding protein and cochemotaxin exhibited identical molecular weights, isoelectric points, antigenic reactivity, and amino acid composition.

Introduction

Activation of the complement system in human serum results in the generation of the biologically active peptide, C5a (1). C5a is a potent chemoattractant for human PMN (1–3). Upon generation, however, C5a is rapidly converted by the serum enzyme, carboxypeptidase N, to the less potent peptide, C5a des Arg (2, 4). C5a des Arg is $\sim$ 10–20-fold less active than C5a as a chemotactic factor for PMN (3, 5, 6).

Previously, we reported that the chemotactic activity of highly purified human C5a des Arg is enhanced significantly by an anionic polypeptide, cochemotaxin, present in normal human serum and plasma (5). More recently, we provided evidence indicating that the cochemotaxin forms a complex with native C5a des Arg, probably by attaching to sialic acid residues present within the oligosaccharide portion of C5a des Arg (6). The cochemotaxin/C5a des Arg complex (molar ratio = 1:2) exhibits potent chemotactic activity for PMN (6). For-

mation of the complex (and its chemotactic activity) was inhibited by the presence of exogenous sialic acid (6). The nature of the cochemotaxin was not elucidated.

We have investigated the nature of the cochemotaxin and, in this report, we present evidence indicating that vitamin D-binding protein (group-specific component [Gc][1] globulin) is the putative C5a des Arg cochemotaxin.

Methods

Preparation of zymosan-treated serum (ZTS), purified human C5a, C5a des Arg, cochemotaxin, and vitamin D-binding protein. Normal human serum was activated with 1.0 mg/ml zymosan in the presence and absence of 1.0 M of the carboxypeptidase N inhibitor, epsilon aminocaproic acid (EACA) as described (5, 6). C5a was isolated from yeast-activated normal human serum containing the carboxypeptidase N inhibitor, 2-mercaptomethyl-3-guanidino ethylthiopropanoic acid (Calbiochem-Behring Corp., La Jolla, CA) (6). Final purification of C5a was accomplished by immunoabsorption chromatography on a column of Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) to which an MAb raised against human C5a was linked covalently. C5a des Arg was prepared by treating the respective C5a preparations (15 µg/ml) with insoluble carboxypeptidase B (Worthington Biochemical Corp., Freehold, NJ), as described previously (5).

The C5a des Arg cochemotaxin was prepared as described previously (5). Final purification was accomplished by HPLC on a protein analysis column (Spherogel SW-3000; Beckman Instruments, Inc., Fullerton, CA) (5, 6).

Affinity-purified human vitamin D-binding protein was the generous gift of Dr. Stuart Lind and Dr. Thomas P. Stossel (Massachusetts General Hospital, Boston, MA). Vitamin D-binding protein was radioiodinated (specific activity = 1.0 × 10⁶ cpm/µg protein) by the lactoperoxidase method, as described (6). Radioiodination did not alter its biologic activity (6).

PMN chemotaxis. Leukocyte suspensions containing 97–99% PMN were prepared from anticoagulated human venous blood by Ficoll/Hypaque centrifugation and dextran sedimentation, as described previously (5, 7). Purified PMN were suspended in Hanks' buffered saline containing 20 mg/ml BSA (Gibco, Grand Island, NY). Directed migration (chemotaxis) of PMN was measured using a minor modification (7) of the leading front method of Zigmond and Hirsch (8). Stimulated random migration in response to albumin-containing buffer (9) and total migration in response to chemoattractants are determined as the distance (micrometers per 35 min) that the leading front of cells migrated into 3.0-µm pore-diameter cellulose nitrate filters (Sartorious Filters, Inc., Hayward, CA) separating the upper, or cell compartments, from the lower, or stimulus compartments, of modified Boyden chambers (Nuclepore Corp., Pleasanton, CA). Che-

Address reprint requests to Dr. H. Daniel Perez, Building 30, Room 3300, San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA 94110.

1. *Abbreviations used in this paper:* EACA, epsilon aminocaproic acid; Gc, group-specific component; 2D-PAGE, two-dimension PAGE; ZTS, zymosan-treated serum.

motaxis (net migration) was calculated by subtracting stimulated random motility from total migration (5, 6). Duplicate chambers were used in each experiment and five fields were examined in each filter.

PAGE. Two-dimension PAGE (2D-PAGE) was performed as described by O'Farrell (10). After electrophoresis, proteins were recovered by transfer to nitrocellulose paper as described by Towbin et al. (11, 12). Immediately after transfer, proteins were reacted with 2.0 μg/ml goat IgG fraction directed against Gc-globulin (Atlantic Antibodies, Scarborough, ME) for 90 min. Nitrocellulose sheets were rinsed and incubated with rabbit anti-goat IgG (Cappel Laboratories, Malvern, PA) for 90 min. Nitrocellulose sheets were rinsed again and incubated with ^{125}I-labeled Staphylococcus protein A (Pharmacia Fine Chemicals) (12). After 90 min, the nitrocellulose sheet was rinsed, wrapped in plastic wrap, and exposed (for 18 h at −70°C) to Kodak XR-1 film, using an intensifying screen (Cronex; DuPont Co., Diagnostic & Bio Research Systems, Wilmington, DE).

Amino acid analysis. Amino acid analysis of purified cochemotaxin and vitamin D-binding protein was performed by using an analyzer (121 MB; Beckman Instruments, Inc.) after hydrolysis of triplicate samples in 6 N HCl for 20 h at 100°C in sealed, evacuated tubes (13).

Other materials. Goat antibody (purified IgG fraction) against alpha₁-acid glycoprotein was purchased from Atlantic Antibodies. *N*-Acetyl-D-glucosamine, mannose, galactose, sialic acid, and EACA were obtained from Sigma Chemical Co., St. Louis, MO. The synthetic chemotactic peptide FMLP was from Peninsula Laboratories Inc., Belmont, CA.

Results

Initially, we performed experiments in which we examined the effect of 100 μg/ml anti–Gc globulin antibodies on the chemotactic activity exhibited by suboptimal (0.1%, vol/vol) concentrations of ZTS. Whereas 0.1% ZTS exhibited significant chemotactic activity (total PMN migration = 82.2±2.2 μm buffer/35 min, 130.3±3.3 μm ZTS/35 min), ZTS that had been treated with anti–Gc antibodies did not (85.6±3.7 μm ZTS + anti–Gc/35 min). Interestingly, anti–Gc globulin antibodies had no effect on the chemotactic activity exhibited by suboptimal (0.05%, vol/vol) concentrations of serum that had been activated with zymosan in the presence of 1.0 M EACA, to prevent conversion of C5a to C5a des Arg. Total PMN migration was 82.2±2.2 μm buffer/35 min, 141.4±2.5 μm EACA-ZTS/35 min, and 138.6±2.5 μm EACA-ZTS + anti–Gc antibodies/35 min. Next, we performed experiments in which we compared the ability of purified vitamin D-binding protein and purified cochemotaxin to enhance the chemotac-

tic activity of purified C5a des Arg (Fig. 1 *A*). Whereas 5.0 ng/ml C5a des Arg did not stimulate directed migration of PMN (6), the same concentration of C5a des Arg exhibited significant chemotactic activity when assayed in the presence of either purified cochemotaxin or vitamin D-binding protein (Fig. 1 *A*). As can be seen, the concentrations of cochemotaxin and vitamin D-binding protein required to enhance the chemotactic activity of C5a des Arg were identical. Furthermore, neither cochemotaxin nor vitamin D-binding protein influenced the chemotactic activity exhibited by suboptimal concentrations of either 1.0 ng/ml C5a (net chemotaxis for C5a alone, 31.2±2.3 μm/35 min; for C5a plus 10 ng/ml cochemotaxin, 32.3±3.1 μm/35 min; and for C5a plus 10 ng/ml vitamin D-binding protein, 32.8±2.9 μm/35 min) or 10^{-9} M FMLP (not shown) (6). Functional evidence that the two proteins were antigenically related was obtained by the use of goat IgG antibodies against Gc globulin (vitamin D-binding protein) (Fig. 1 *A*). 30 min preincubation at 37°C of either cochemotaxin or vitamin D-binding protein (10 ng/ml each) with 10 μg anti–Gc globulin antibodies completely abolished the enhancing effect of these peptides on the chemotactic activity of C5a des Arg. 10 μg/ml anti–Gc antibodies had no effect on the ability of PMN to migrate either randomly (PMN migration to buffer, 92.0±2.8 μm/35 min; buffer plus anti–Gc antibodies, 91.2±3.1 μm/35 min) or in response to chemotactic concentrations of 40 ng/ml C5a des Arg (C5a des Arg alone, 122.9±3.7 μm/35 min; C5a des Arg plus anti–Gc antibodies, 123.8±3.3 μm/35 min). The effect of the antibody was specific, since similar concentrations of goat IgG antibodies against alpha₁-acid glycoprotein had no effect (not shown).

Evidence that the cochemotaxin and vitamin D-binding protein may interact with C5a des Arg in a similar manner was obtained from experiments in which we examined the ability of various sugars (i.e., components of the oligosaccharide chain of C5a des Arg) to interfere with the enhancing effect of vitamin D-binding protein on the chemotactic activity of C5a des Arg (Fig. 1 *B*). Sialic acid inhibited the enhancing effect of vitamin D-binding protein in a concentration-dependent fashion. The degree of inhibition by increasing concentrations of sialic acid was similar to that reported for the cochemotaxin (6). The inhibitory effect of sialic acid was specific, since it was not observed when other components of the oligosaccharide chain of C5a des Arg were used (Fig. 1 *B*).

To determine if vitamin D-binding protein interacted with C5a des Arg, we performed molecular sieve chromatography
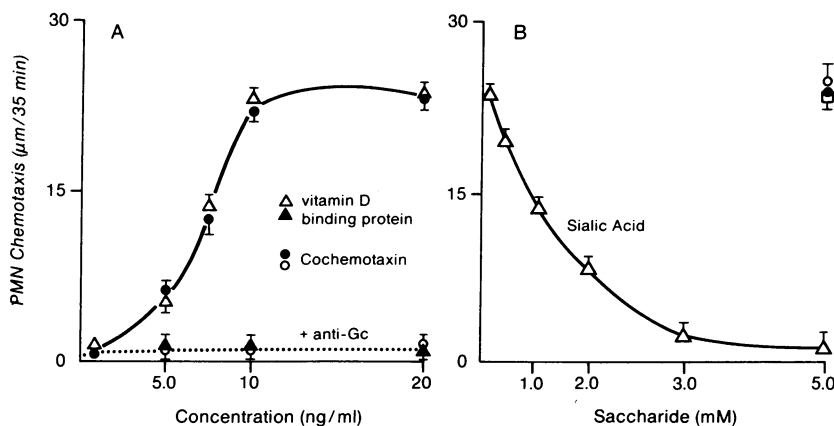
Figure 1. (*A*) Enhancement of the chemotactic activity of 5.0 ng/ml native C5a des Arg by increasing concentrations of either vitamin D-binding protein (△) or cochemotaxin (●). The effect of 10 μg/ml anti–vitamin D-binding protein IgG on the ability of either vitamin D-binding protein (▲) or cochemotaxin (○) to enhance the chemotactic activity of 5.0 ng/ml native C5a des Arg. (*B*) Effect of increasing concentrations of various sugars on the chemotactic activity exhibited by a mixture of 5.0 ng/ml C5a des Arg and 10 ng/ml vitamin D-binding protein. △, sialic acid; ○, galactose; ●, mannose; and □, *N*-acetyl-D-glucosamine.

on Bio-Gel P-150 (Bio-Rad Laboratories, Richmond, CA). [^{125}I]Vitamin D-binding protein was applied to a 1.0 × 20 cm column of polyacrylamide gel and eluted with HBSS (pH 7.4). 0.3-ml fractions were collected and radioactivity was determined. When chromatographed alone, 10 ng [^{125}I]vitamin D-binding protein eluted as a symmetric peak, exhibiting an apparent molecular weight of 60,000 (Fig. 2 *A*). When a mixture of 10 ng [^{125}I]vitamin D-binding protein and 5.0 ng C5a des Arg were chromatographed under identical conditions, the peak of radioactivity eluted with an apparent molecular weight of 80,000 (Fig. 2 *B*), an effect that was prevented by the presence of 5.0 mM sialic acid (Fig. 2 *B*) (6). Analysis of purified cochemotaxin and vitamin D-binding protein by 2D-PAGE and Western blot revealed that both proteins had three isoforms, which exhibited identical molecular weights and isoelectric points (Fig. 3, *A* and *B*), as well as antigenic reactivity with anti–Gc globulin antibodies (Fig. 3, *C* and *D*).

Finally, amino acid analysis of these two peptides revealed an almost identical composition (Table I).

Discussion

The results presented here indicate that vitamin D-binding protein is functionally identical to the previously described C5a des Arg cochemotaxin (Figs. 1 and 2) (6). Vitamin D-binding protein specifically enhances the chemotactic activity of C5a des Arg (Fig. 1 *A*), probably by attaching (i.e., complex formation) to sialic acid residues within the oligosaccharide chain of native C5a des Arg (Figs. 1 *B* and 2). The biologic activities of the C5a des Arg cochemotaxin and vitamin D-binding protein are blocked, specifically, by anti–Gc globulin antibodies (Fig. 1 *A*).

Evidence that the two peptides are related was obtained by 2D-PAGE and Western blot (Fig. 3). Isoforms of vitamin D-binding protein and the cochemotaxin exhibited identical molecular weight ($\cong$ 58,000) and isoelectric points (4.9, 5.16, and 5.3, respectively) and reacted with anti-Gc globulin. Isoforms of Gc globulin have been demonstrated previously (14). Furthermore, the two peptides exhibit identical amino acid com-
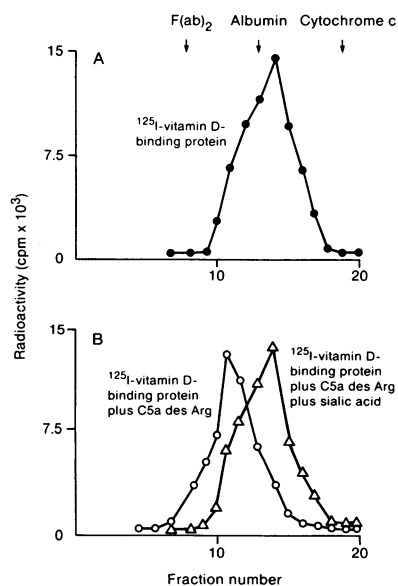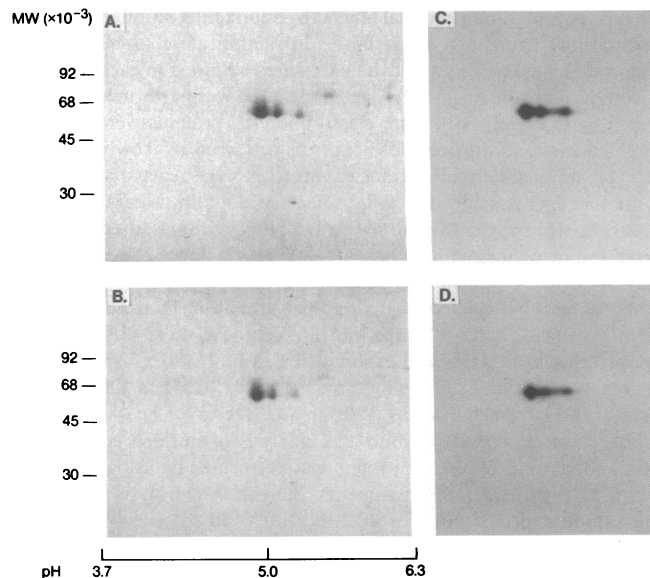
Figure 3. Coomassie Blue stain (2D-PAGE) of (*A*) vitamin D-binding protein and (*B*) cochemotaxin. Western blot analysis of 2D-PAGE of (*C*) vitamin D-binding protein and (*D*) cochemotaxin. Approximately 3.0 μg of each protein was used, and both proteins were run simultaneously in the first and second dimension. Second dimension was performed using 7–17% polyacrylamide gradient slab gels.

position (Table I), supporting the notion that vitamin D-binding protein and cochemotaxin are identical.

The precise mechanism by which vitamin D-binding protein (cochemotaxin) enhances the chemotactic activity of C5a

Table I. Amino Acid Composition of Vitamin D-binding Protein and Cochemotaxin

Amino acid*	Vitamin D-binding protein	Cochemotaxin
	mol %	
Lysine	8.7	8.1
Histidine	1.7	1.72
Arginine	3.4	3.4
Aspartic acid	9.4	9.4
Threoine	7.1	7.0
Serine	8.7	8.6
Glutamic acid	11.6	11.8
Proline	6.1	6.0
Glycine	4.6	4.5
Alanine	7.1	7.0
Cystine (half)	3.2	3.4
Valine	6.5	6.4
Methionine	1.4	1.4
Isoleucine	2.3	2.3
Leucine	10.9	10.8
Tyrosine	3.5	3.5
Phenylalanine	4.2	4.2
Tryptophan	ND‡	ND‡

* Results are the averages of two determinations obtained after 20 h hydrolysis.
‡ ND, not done.

Figure 2. Elution profiles of radioactivity after molecular sieve chromatography of (*A*) 10 ng/ml [^{125}I]vitamin D-binding protein; (*B*) 10 ng/ml [^{125}I]vitamin D-binding protein plus 5.0 ng/ml native C5a des Arg (○); and 10 ng/ml [^{125}I]vitamin D binding-protein plus 5.0 ng/ml native C5a des Arg plus 5.0 mM sialic acid (△).

des Arg is, at present, unclear. One possible explanation is that it masks the oligosaccharide side chain of C5a des Arg (6). Gerard et al. (15) have reported that removal of the oligosaccharide chain of C5a des Arg, but not C5a, enhances its chemotactic activity. We have confirmed those findings (6) and demonstrated that the cochemotaxin has no effect on the chemotactic activity of deglycosylated C5a des Arg (6). Alternatively, vitamin D-binding protein may enhance the binding of C5a des Arg to its PMN receptor. Finally, by complexing with C5a des Arg (molar ratio vitamin D-binding protein/C5a des Arg, 1:2), vitamin D-binding protein may increase the number of receptors occupied by C5a des Arg per surface area (i.e., increased concentration of stimulus per unit of PMN membrane).

Furthermore, it is of interest to determine if the enhancing effect of vitamin D-binding protein is a property of a single isoform. Since isoforms vary from species to species (14), this phenomenon may explain why certain sera (i.e., porcine) (6) do not enhance the chemotactic activity of human C5a des Arg. These possibilities are now under investigation.

Acknowledgments

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