Brief Definitive Report

Identification of C3 β Chain as the Human Serum Eosinophil Cytotoxicity Inhibitor

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Summary

An eosinophil cytotoxicity inhibitor (ECI) was purified from serum of a human subject with severe allergic dermatitis. Molecular weight of the isolated polypeptide (75,000) and its NH_2-terminal amino acid sequence identified it as the β chain of the C3 complement component (apparently free, but perhaps attached to very small fragments of the α chain). Free β chain, prepared from normal plasma by reduction of C3, inhibited both eosinophil cytotoxicity and neutrophil adherence functions, with half-maximal activity at ~250 ng/ml. Apparently free C3 β chain was detected in certain human biological fluids associated with inflammation; the presence of C3 β chain correlated with ECI activity. This evidence demonstrates a potential role for free C3 β chain as a suppressor of eosinophil and neutrophil functions in inflammation.

Serum from a small percentage of recently studied human subjects, including three with chronic inflammation, contained a ~80-kD polypeptide (eosinophil cytotoxicity inhibitor [ECI]) that suppressed the cytotoxic function of TNF- or granulocyte/macrophage (GM)-CSF-stimulated eosinophils. Normal human serum contained an inactive precursor of ECI that could be activated artificially by certain chromatographic procedures. It was hypothesized that, in vivo, ECI is activated by some property of inflammation (1).

Here we describe the purification of ECI from serum of a human subject with severe allergic dermatitis. We show that this substance is structurally similar and probably identical to the β chain of the C3 complement component, that free β chain prepared from normal plasma has the activity of ECI, and that apparently free C3 β chain exists in certain human biological fluids associated with inflammation.

Materials and Methods

Isolation of ECI. The following protocol was developed, based on the screening of fractions with eosinophil cytotoxicity and neutrophil adherence assays (see below). Fractions containing the inhibitor did not affect eosinophil or neutrophil viability, as judged by trypan blue exclusion and H_2O_2 release after PMA stimulus (1). Serum from a human subject with severe allergic dermatitis was brought to 50% saturation with ammonium sulfate at 0°C overnight and centrifuged at 12,000 *g*. The supernatant portion was brought to 65% saturation, reincubated, and centrifuged as above. The resulting pellet was dissolved and dialyzed in 0.04 M sodium phosphate buffer, pH 7.2, and then incubated with controlled pore glass beads (1:100 [vol/vol]; Electronucleonics, Fairfield, NJ). The beads were washed with 20% and then 30% ethylene glycol in the same buffer. Material from the 30% wash was subjected to dialysis (H_2O), lyophilization, and applied to a TSK-Phenyl 5PW 7.5 × 75-mm column (HPLC). Bound proteins were eluted with a 45-min gradient of 0.6 M ammonium sulfate in 100 mM phosphate, pH 7.0., to 30% ethylene glycol in 10 mM phosphate, pH 7.0. The material collected from 28–31 min of the gradient was dialyzed in 0.02 M Tris, pH 7.0, and applied to a TSK-DEAE 5PW 7.5 × 75-mm column. Bound material was eluted with a 45-min gradient to 0.5 M ammonium sulfate in the same buffer. The material collected from 10–13 min of this gradient was collected and applied to a Vydac C-4 4.6 × 250-mm column, washed with 0.1% trifluoroacetic acid, and eluted with a 30-min gradient to 50% acetonitrile in 0.1% trifluoroacetic acid. The active material was recovered at 19 or 20 min of this gradient.

Biological Assays. Human eosinophils, isolated at 80–99% purity (2), were tested for the ability to kill larvae of *Schistosoma mansoni* in the presence of antibody (3) and a test or control source of inhibitor. The eosinophils were treated with either 100 U/ml of purified recombinant TNF (Cetus Corp., Emeryville, CA) or 20 pM purified recombinant GM-CSF (Dr. Judith C. Gasson, UCLA School of Medicine) in order to activate the killing functions (4, 5).

Neutrophils were isolated at >95% purity by dextran sedimentation, centrifugation through a cushion of Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ), and hypotonic lysis to remove remaining erythrocytes. For assay of adherence to tissue culture plastic, 10^5 neutrophils were added to tissue culture plate microwells (Falcon Labware, Oxnard, CA) in 200 μl of medium (RPMI, pH 7.2; 100 U/ml penicillin G; 100 μg/ml streptomycin; 8% NuSerum [Collaborative Research, Waltham, MA]; and 2% HIFCS) containing 100 U/ml recombinant TNF and a test or control source of inhibitor. The neutrophils were allowed to adhere at 37°C for

2 h, and then nonadherent cells were removed by washing with warm saline. The wells were emptied of liquid and filled with 200 μl of a solution containing 0.1% Triton X-100, 0.4 mM 2-2′ azino-di-3-ethyl-benzthiazoline-6-sulfonate plus 0.003% hydrogen peroxide in 0.05 M sodium citrate, pH 4.0. The generation of colored product (absorbance monitored at 405 nm) reflected the quantity of cell-derived myeloperoxidase in the wells and correlated with visual assessments of cell adherence.

Amino Acid Sequence Analysis. After tentative identification as ECI, the 75-kD polypeptide species was isolated in quantities suitable for amino acid sequence analysis after size exclusion HPLC of allergic dermatitis serum. The fraction containing ECI activity was separated by SDS-PAGE (10% polyacrylamide under reducing conditions). Polypeptide species were then transferred to PVDF membrane and stained with coomassie blue. Material from SDS-PAGE run in parallel was recovered by electroelution of gel slices. These samples were dialyzed in medium and tested for the ability to inhibit eosinophil cytotoxic function. The region of PVDF membrane containing the polypeptide species that was associated with inhibitory activity was then excised and submitted for amino acid sequence analysis (performed by Margaret Ehrhardt, Brigham and Women's Hospital core sequencing facility, using a Gas Phase Protein Sequencer [475A; Applied Biosystems, Inc., Foster City, CA]). Computer search was carried out for a translated version of GenBank data base 61.

Preparation of C3 Fragments. C3 was prepared from normal human plasma according to reference 6. For separation of the β chain, C3 was reduced in 10% 2-ME for 1 h at room temperature. To alkylate sulfhydryl groups, portions of the reduced material were mixed with an equal volume of 0.75 M iodoacetamide on ice for 1 h. Free β chain was isolated by preparative SDS-PAGE under reducing conditions and electroelution in 0.05 M ammonium bicarbonate. Eluted protein and similarly processed material from other regions of the gels (control samples) were dialyzed in medium. C3c was prepared by trypsin digestion of C3 (7) and analyzed by SDS-PAGE. Protein concentrations were determined by the Bradford method (reagents from Pierce Chemical Co., Rockford, IL).

Detection of C3 β Chain by Western Blot Analysis. 1-μl samples of biological fluids were separated by SDS-PAGE (nonreducing conditions)/Western blot analysis (8), using a peroxidase-conjugated second antibody (anti-mouse IgG,A,M; diluted 1/10,000; Cappel Laboratories, Malvern, PA). After electrophoretic transfer to PVDF membrane, the resolved species were detected by treatment with pooled culture supernatants from four β chain–specific hybridoma lines.

The hybridomas were generated after immunization of BALB/c mice with ~100 μg of purified C3 β chain in CFA and subsequently IFA. Spleen cells were fused with SP2 myeloma cells and cultured in selective medium according to standard methods. Hybridoma culture supernatants were screened by ELISA and Western blot analysis for reactivity with free C3 β chain in reduced preparations of purified C3. Positive hybridoma lines were subcloned twice.

Results and Discussion

ECI was purified from serum of a human subject with severe allergic dermatitis by a sequence of methods including: high salt precipitation, controlled pore glass bead chromatography, hydrophobic interaction HPLC, anion exchange HPLC, and reversed-phase HPLC. Two biological assays, inhibition of human eosinophil cytotoxic function and of human neutrophil adherence to tissue culture plastic, were used to evaluate samples generated by chromatography. These methods allowed the tentative identification of ECI as a 75-kD species (Fig. 1). During all steps of the purification, there was approximate agreement between the eosinophil-based and neutrophil-based assays, suggesting that the inhibitory activity affects both leukocyte types.

A partial NH_2-terminal amino acid sequence for purified ECI is shown below. Computer search of known polypeptide sequences revealed strong homology of ECI to the β chain of the C3 complement component (sequences aligned below).

Purified ECI: ? -Pro-Met-Tyr-Ser-Ile- ? -Thr-Pro-Asn-
C3 β chain: Ser-Pro-Met-Tyr-Ser-Ile-Ile-Thr-Pro-Asn-

? - ? - ? -Leu-Glu-Ser- ? -Glu- ? -Met . . . .
Ile - Leu - Ala -Leu-Glu-Ser- Glu -Glu- Thr -Met . . . .

The apparent molecular mass of ECI (75 kD) was also in agreement with the known mass of the C3 β chain. This suggests that ECI is free β chain, but does not rule out the possibility that it is attached to very small fragments of the α chain.

To test the relationship between ECI and C3 β chain by an alternative method, we studied activity of the following substances, all purified from normal human plasma (see Materials and Methods): C3, C3 treated with 2-ME to separate the α and β chains, C3 reduced and alkylated with iodoacetamide to prevent possible reassociation of the α and β chains, and free β chain.

Intact C3 had no significant inhibitory activity at concentrations as high as 120 μg/ml of protein. Reduction of C3, and particularly reduction and alkylation, to generate free β chain produced substantial inhibitory activity. Free β chain exhibited the greatest activity (half maximal at ~250 ng/ml), though a mock preparation from an adjacent SDS-PAGE slice had negligible activity. Alkylation did not affect the activity of free β chain, demonstrating the absence of a functional role for the reduced sulfhydryl groups (Fig. 2).

As C3c (C3 β chain attached to 43- and 27-kD fragments of the α chain by disulfide bonds) is generally considered the terminal cleavage product of C3 during the activation of complement (10, 11), we wanted to learn whether C3c had inhibitory activity similar to free β chain. C3c was prepared by trypsin digestion of C3 and tested in assays of eosinophil cytotoxic function and neutrophil adherence. This material exhibited no detectable inhibitory activity (data not shown).

Several clinically interesting biological fluids were tested for the presence of C3 β chain by Western blot analysis of SDS-PAGE gels (nonreducing conditions). Apparently free β chain was detected in plasma drawn from a human subject during a severe allergic dermatitis reaction and in synovial fluid of two patients with rheumatoid arthritis (one example shown), but not in normal plasma (Fig. 3 *A*). The presence of free β chain correlated with the ability of these biological fluids to inhibit eosinophil cytotoxic function (comparison of Fig. 3, *A* and *B*).

Control experiments indicated that levels of β chain in samples were not affected substantially by the process of coagu-

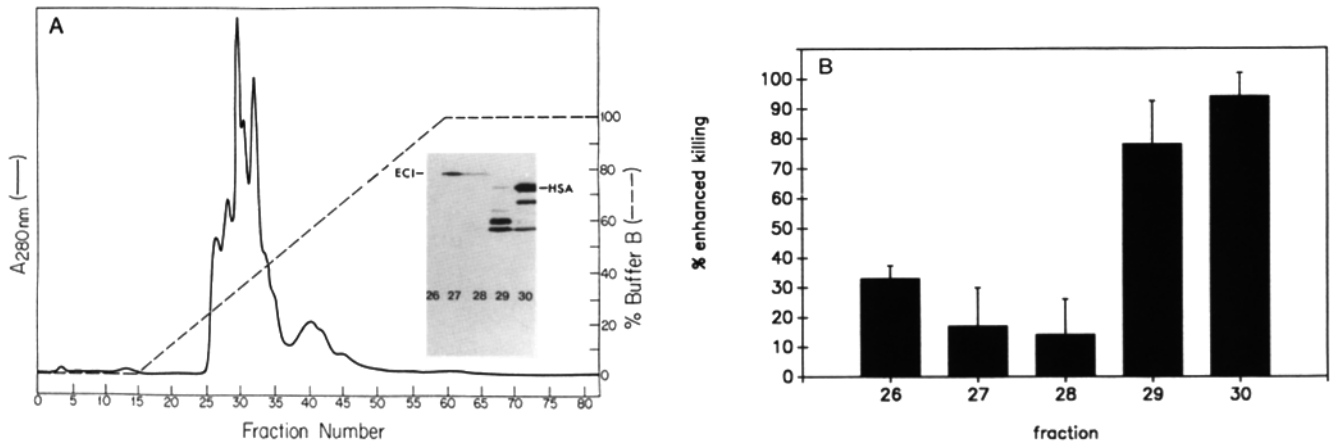

Figure 1. Tentative identification of ECI as a 75-kD peptide. (*A*) An example of the anion exchange HPLC step. The elution gradient and profile of eluted polypeptides are indicated. The inset shows the SDS-PAGE analysis of the fractions assayed for activity in *B*, with purified ECI and (for comparison) human serum albumin indicated. (*B*) Effect of chromatography fractions on eosinophil cytotoxic function. The data represent means (plus SE) from five experiments with eosinophils obtained from different donors. The 100% value represents cytokine-stimulated (in this case, 100 U/ml of TNF, however, similar results were obtained with 20 pM GM-CSF) eosinophil cytotoxic function or neutrophil adherence. The 0% value represents the low or negligible functions (which were subtracted as background) in the absence of cytokine stimulation. Note the presence of the 75-kD polypeptide in fractions (primarily fractions 27 and 28) with inhibitory activity.

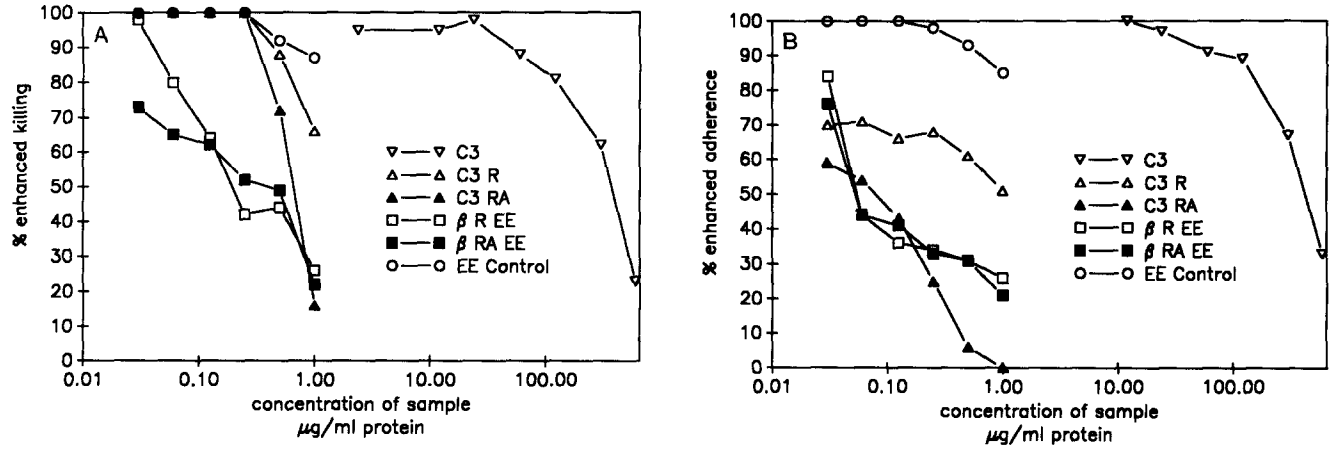

Figure 2. Inhibition of eosinophil and neutrophil function by free C3 β chain. C3 (▽), reduced C3 (△), reduced and alkylated C3 (▲), reduced (□), or reduced and alkylated (■) β chain recovered by electroelution from SDS-PAGE, and a mock preparation electroeluted from a reducing SDS-PAGE gel (○) were tested for inhibition of eosinophil cytotoxic function (*A*) and neutrophil adherence (*B*).

lation or the choice of anticoagulant method. Furthermore, activation of complement by zymosan treatment did not generate β chain or its inhibitory activity, as evaluated by Western blot analysis and bioassays (data not shown). To this point, our work does not provide indications regarding the molecular mechanisms or specific disease processes that generate the β chain inhibitory substance.

The stepwise cleavage of C3 to C3c during the activation of complement generates C3a, C3b, C3bi, and C3dg, which all enhance leukocyte functions in the development of inflammation (10–14). For example, C3b or C3bi enhance eosinophil functions, including cytotoxicity (12, 13), and promote neutrophil functions, including adherence (10–14). Here, we present evidence that C3 β chain inhibits these functions and that apparently free β chain exists as a discreet entity in biological fluids associated with inflammation. Taken collectively, this evidence demonstrates a potential role of C3 β chain as a feedback mechanism to suppress leukocyte functions in inflammation.

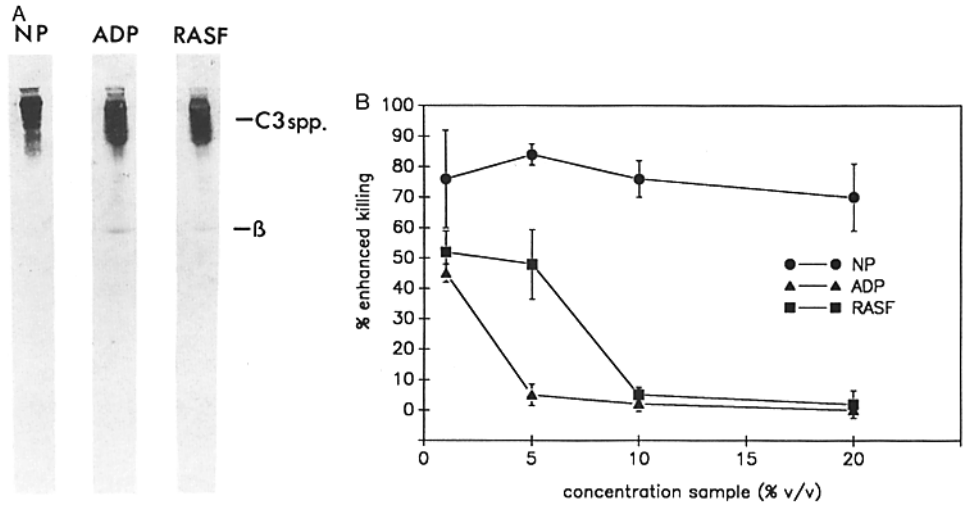

Figure 3. Presence of free C3 β chain in selected human biological fluids. Normal plasma (●), plasma from an allergic dermatitis patient (▲), and synovial fluid from a rheumatoid arthritis patient (■) were analyzed. (*A*) Western blot analysis indicating the presence of β chain in various C3 forms (C3 spp.) in all samples and free β chain only in ADP and RASF. (*B*) Inhibitory activity of these biological fluids with respect to eosinophil cytotoxic function. Note that inhibition correlated with the presence of free β chain.

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