A SERUM FACTOR THAT SUPPRESSES THE CYTOTOXIC FUNCTION OF CYTOKINE-STIMULATED HUMAN EOSINOPHILS

By DAVID S. SILBERSTEIN,* MARJORIE S. MINKOFF,* ABLA A. CREASEY,‡
AND JOHN R. DAVID*§

*From the *Department of Medicine, Harvard Medical School and the Department of Rheumatology
and Immunology, Brigham and Women's Hospital, Boston, Massachusetts 02115; the ‡Department
of Cell Biology, Cetus Corporation, Emeryville, California 94608; and the §Department of Tropical
Public Health, Harvard School of Public Health, Boston, Massachusetts 02115*

The monokine known as tumor necrosis factor or cachectin (TNF) is a pro-inflammatory immunological mediator, with the ability to stimulate in vitro functions of human eosinophils (1, 2), neutrophils (3–7), monocytes (8), endothelial cells (4, 9, 10), and fibroblasts (11, 12).

In an earlier study (1) we found that TNF increased the aggregation and adherence to schistosomula (*Schistosoma mansoni*) of neutrophils obtained from all but 1 of 12 blood donors. Subsequent experiments with both eosinophils and neutrophils from that single human subject (NR, for nonresponder) are described here. These cells were usually unresponsive to TNF in assays of antibody-dependent toxicity to schistosomula, aggregation, and adherence to plastic, or generation of hydrogen peroxide in response to stimulus with aggregated IgG.

During studies of the mechanism underlying the apparent unresponsiveness to TNF, we detected a factor in NR serum that inhibits the cytotoxic function of TNF-stimulated eosinophils. This substance has been designated ECI (for eosinophil cytotoxicity inhibitor).[1] This article describes physical and functional characteristics of ECI as well as evidence that ECI can be derived from an inactive precursor in normal human serum.

Materials and Methods

Cytokines. Recombinant human TNF, produced by *Escherichia coli* and purified to near homogeneity (>90%, judged by SDS-PAGE), containing <2 ng/mg endotoxin (assayed by LAL Assay Associates of Cape Cod, Inc., Woods Hole, MA) (13), was provided by the process development group at Cetus Corporation.

Unless otherwise specified, TNF was used at a concentration of 100 U/ml, the optimal dose for the eosinophil cytotoxicity assay (1). For some experiments, purified mAb 245-10E11 (14) was used to neutralize the activity of TNF. The neutralizing titer of the antibody was determined by the L929 cell cytotoxicity assay (see below).

---

[1] *Abbreviations used in this paper:* ECI, eosinophil cytotoxicity inhibitor; GM-CSF, granulocyte/macrophage colony-stimulating factor.

Recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF), produced by COS cells and purified, was provided by Dr. Judith C. Gasson (Division of Hematology and Oncology, Department of Medicine, UCLA School of Medicine, Los Angeles, CA). GM-CSF was used at a final concentration of 20 pM, the optimal dose in the eosinophil cytotoxicity assay (15).

Preparation of Human Serum, Eosinophils, and Neutrophils.   Venous blood was obtained from laboratory personnel for the preparation of serum, eosinophils and neutrophils. Some of the donors for eosinophil studies had moderately elevated eosinophil counts in association with allergies and/or asthma (total eosinophil counts ranged from 1 to 18% of total leukocytes for these studies). The subject whose cells failed to respond to TNF (NR) was also a laboratory staff member with a history of severe delayed-type hypersensitivity dermatitis reactions.

Blood for the preparation of serum was collected in glass tubes, incubated at 37° for 1 h, and then overnight at 4°. The serum was clarified by centrifugation and stored frozen at -20°.

Eosinophils were isolated by a sequence of dextran sedimentation and discontinuous metrizamide density gradient centrifugation (16). Erythrocytes were removed by hypotonic lysis. The purity of eosinophil preparations, as evaluated by examination of Wright's-Giemsa stained slides, ranged from 80 to 99%, with neutrophils as the contaminating cells.

Neutrophils were prepared by a sequence of dextran sedimentation and Ficoll-Hypaque centrifugation (17). Erythrocytes were removed by hypotonic lysis. The neutrophil preparation were essentially free of mononuclear cells and contained <10% eosinophils.

Two serum samples from patients with hypereosinophilia (one with idiopathic hypereosinophilic syndrome and one with eosinophil infiltration of the lingual/buccal tissues) were provided by Dr. William F. Owen (Department of Rheumatology and Immunology, Brigham and Women's Hospital, Boston, MA).

100 anonymous serum samples were supplied by the American Red Cross Blood Services, Northeast Region (Dedham, MA; special thanks to Dr. Mark A. Popovsky and to Celeste LeFort), for use in studies related to Fig. 2 and Table I. Pooled human type AB serum (lot no. 29309039) was obtained from Flow Laboratories (McLean, VA).

Human plasmas deficient in coagulation factors XI, XII, prekallikrein, or high molecular weight kininogen were obtained from George King Biomedical, Inc. (Overland Park, KS). The plasmas were recalcified to a final concentration of 0.4 M $CaCl_2$, and the clots that formed were removed by centrifugation. Control experiments with normal sera and recalcified plasmas showed that this procedure did not affect the functional properties of the serum inhibitor (ECI).

Lipoprotein-free human serum and purified high density, low density, and very low density lipoproteins were obtained from Sigma Chemical Co. (St. Louis, MO). Antibody to schistosomula of *S. mansoni* was obtained from Brazilian schistosomiasis patients, as described previously (1).

Assay of Eosinophil or Neutrophil Cytotoxic Function.   Antibody-dependent killing of *S. mansoni* larvae was measured as described previously (1), using heat-inactivated serum from Brazilian schistosomiasis patients as a source of antibody. Effector cells (E/T ratio of 1,000:1 for eosinophils and 5,000:1 for neutrophils), antibody (diluted 1/100), schistosomula targets (100/well), and cytokine were cultured (medium: MEM with Earle's salts, supplemented with 25 mM Hepes, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS serum [all from Gibco Laboratories, Grand Island, NY]) for 36-40 h. After this period, killing was scored by microscopy. Control or test samples of inhibitors were added to the cultures at various times in an additional volume of 50 μl, usually dissolved or dialyzed in medium. For experiments in which the inhibitor was removed, the contents of microwells were transferred to 12 × 75 mm polypropylene tubes at various times, diluted with 5 ml of medium, and centrifuged at 500 g for 5 min. The supernatant was aspirated, and the eosinophils and schistosomula in the pellet were recultured in 250 μl of assay medium with fresh antibody and cytokine.

Assay of Eosinophil and Neutrophil Adherence to Plastic.   Eosinophils or neutrophils were suspended at a concentration of 10^5 cells/200 μl of assay medium in the presence or absence of 100 U/ml TNF and allowed to settle in round-bottomed microtiter wells at 37°C for 2 h. After this period, the adherence of eosinophils to the plastic surface was assayed by one of two methods. As described previously (1), using an inverted microscope the wells were exam-

ined for the presence of adherent cells covering the sides of the wells. In the absence of TNF, there was very little adherence of eosinophils or neutrophils to the plastic, and most of the cells were in a pellet at the bottom of the wells. As an alternative method of measuring the adherence to plastic, nonadherent cells were washed away with warm saline, and 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 pH 4.0, 0.05 M sodium citrate was added to the wells. The generation of colored product (absorbance monitored at 405 nm) reflected the quantity of cell-derived peroxidase in the wells.

Measurement of Eosinophil and Neutrophil Production of H_2O_2. Production of H_2O_2 was measured by quenching of scopoletin fluorescence (18), with purified cells washed and resuspended in buffer (4 mM sodium phosphate, pH 7.4, 128 mM sodium chloride, 12 mM potassium chloride, 1 mM calcium chloride, 2 mM magnesium chloride, and 2 mM glucose) at a concentration of 10^6/ml. The cells (2 ml) were then placed in a cuvette (10 × 10 × 48 mm acrylic; Sarstedt, Princeton, NJ) with 5 μl of horseradish peroxidase stock (6.25 mg/ml buffer, Sigma Chemical Co.), 10 μl of scopoletin stock (1 mM in buffer, Sigma) and 10 μl of control buffer or TNF (for some experiments); they were maintained at 37°C. Fluorescence was monitored at 460 nm (with excitation at 350 nm) by a fluorescent spectrophotometer (No. MPF-44B; Perkin-Elmer, Norwalk, CT). When baseline fluorescence had been established for 5 min, the cells were stimulated either with 10 μl of phorbol myristate acetate (1 mg/ml in ethanol) or 10 μl of IgG aggregates (10 mg/ml). The decrease in fluorescence, which is proportional to the production of H_2O_2, was recorded by a Perkin-Elmer model 056-1001 recorder.

To prepare IgG aggregates, chromatographically purified human IgG (Organon Teknika-Cappel, Malvern, PA) was dissolved in H_2O_2 assay buffer at a concentration of 10 mg/ml. This solution was heated in a water bath at 62°C for 25 min. Control preparations of IgG that were not heat-aggregated did not stimulate detectable production of H_2O_2 by eosinophils or neutrophils.

Assay of TNF Toxicity to L929 Cells. The toxicity of TNF to L929 cells was assayed as described (13). The cells were cultured either with 1 μg/ml actinomycin D for 16 h (seeded initially at 10^5 cells/microwell) or without actinomycin D for 48 h (seeded originally at 2 × 10^4 cells/microwell). The viability of L929 cells was determined by the spectrophotometric measurement of the reduction of the tetrazolium salt MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to formazan by the mitochondrial enzyme succinate dehydrogenase, a reaction that occurs only in living cells (19).

HPLC. For molecular size analysis and partial purification of ECI, 100-μl samples of serum were chromatographed on a TSK-400 BioSil 7 × 600 mm column (Bio-Rad Laboratories) with a solvent consisting of 0.9% sodium chloride, 25 mM Hepes at pH 7.2, 100 U/ml penicillin G, and 100 μg/ml of streptomycin. The column was calibrated using purified human serum albumin, IgG, and IgM, as well as the identifiable peaks of these substances resolved from human serum.

For anion-exchange separations, the sample was injected on a DEAE-5PW 7.5 × 75 mm column (Bio-Rad Laboratories). The column was washed for 15 minutes with 0.02M Tris, pH 8.0, eluted with a 45-min gradient to Tris buffer plus 0.5 M ammonium sulfate, and washed for an additional 15 min with Tris/ammonium sulfate, all at a flow rate of 1 ml/min. Eluted fractions to be assayed for ECI activity were dialyzed in assay medium.

Heat or Trypsin Treatment of ECI. Active fractions from the HPLC sizing analysis (see Fig. 3) were pooled, and aliquots were treated with heat (80°C water bath for 1 h) or treated with trypsin (TPCK-treated trypsin, type XIII, Sigma Chemical Co.; trypsin treatment consisted of two 30-min incubations with 0.5% trypsin at 37°C). Soybean trypsin inhibitor (type I-S, Sigma) was added at fourfold excess neutralizing units either before or after trypsin treatment.

Results and Discussion

Apparent Unresponsiveness of NR Eosinophils and Neutrophils to TNF. Stimulation of normal eosinophils with 100 U/ml of TNF enhanced their antibody-dependent killing of schistosomula from 14.3 ± 8.4% to 45.5 ± 16.8% of targets killed (40 experi-

ments with eosinophils purified from 21 different donors, $p < 0.001$ by the two-tailed t-test, factor of increase ranged from 64 to 1,150%). By comparison, NR eosinophils failed to respond to TNF at doses up to 10,000 U/ml in 9 of 12 parallel experiments (19.3 ± 9.1% killed without TNF; 19.0 ± 7.7% killed with 100 U/ml TNF, for example). In the other three experiments, which were conducted when NR was experiencing different episodes of severe inflammation due to bruising or allergic dermatitis, the cytotoxic function of unstimulated NR eosinophils was elevated (42.7 ± 12.1% killed), and there was also a detectable response to TNF (increase to 63 ± 20.3% killed).

Neutrophils from NR were similarly unresponsive to TNF, as judged by the failure to aggregate and adhere to plastic (12 of 13 experiments), the failure to increase the killing of schistosomula (3 experiments), and the failure to release increased quantities of H_2O_2 following stimulus with aggregated IgG (5 experiments).

The unresponsiveness to TNF was not associated with use of drugs or medications, unusual habits of diet, exercise, or sleep, or any known infections. In view of the occasional responsiveness to TNF in association with inflammation, TNF unresponsiveness by NR eosinophils appeared to result from a regulatory mechanism, rather than a constitutive defect (such as a genetically defective TNF receptor).

Presence of a Factor (ECI) in NR Serum that Reduced the Cytotoxic Function of Normal TNF-stimulated Eosinophils. In the eosinophil cytotoxicity assay, the addition of NR serum (at the time that eosinophils and schistosomula were mixed) suppressed the killing of schistosomula targets by normal TNF-stimulated eosinophils. This effect was half-maximal at a serum concentration of 2–5% and maximal at >10%. Generally, other human sera did not have this effect (Fig. 1). However, inhibitory activity (ECI) was also detectable in a sample of pooled human (type AB) serum (a tissue culture reagent purchased from Flow Laboratories) and in samples from two patients with extreme hypereosinophilia (one with the idiopathic hypereosinophilic syndrome and one with eosinophil infiltration of the lingual/buccal tissues).

To gain further information about the population distribution of the inhibitory activity identified in NR serum, 22 sera from lab personnel and 100 anonymous sera from the Red Cross were tested in the eosinophil cytotoxicity assay. 11 of these
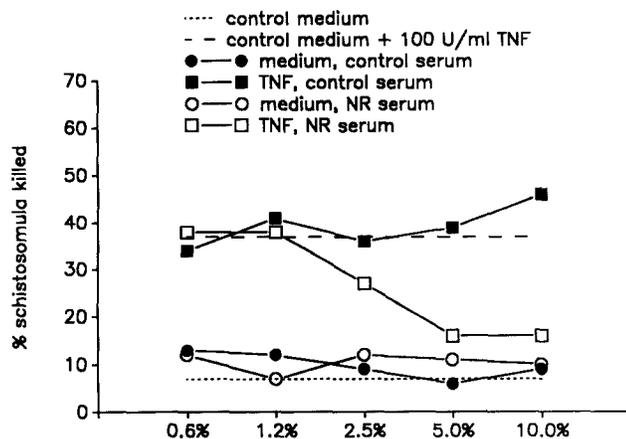

FIGURE 1. The ability of heat-inactivated NR serum to cross-inhibit the response of normal eosinophils to TNF. The cytotoxic function of eosinophils was assayed in the presence or absence of 100 U/ml of TNF. Normal serum or NR serum was added to the cultures at the indicated concentrations. The horizontal lines indicate the level of killing with no serum added in the presence (---) or absence (···) of TNF.

sera reduced TNF-enhanced killing of targets by >50%, and 4 sera reduced TNF-enhanced killing by >90% (Fig. 2).

Physical Characteristics of the Inhibitory Component (ECI) in NR Serum. The molecular weight of the inhibitory component was investigated by chromatography of NR serum on HPLC sizing columns. When the serum was heat-inactivated (56°C for 30 min, necessary for subsequent bioassay) before sizing, the molecular weight appeared to range from 80,000 up to the void volume of the column (>900,000) (not shown). When fractions were heat-inactivated after sizing, the molecular weight appeared to be ~80,000 to 100,000 (Fig. 3). Samples of seven different control sera, with no detectable titers of inhibitor, were chromatographed in a similar manner. Surprisingly, inhibitor of approximately the same molecular weight was detected (example in Fig. 3).

Titration experiments showed that the activity of the inhibitor was increased by the molecular sizing procedure. In the case of whole sera with detectable ECI, the activity increased by a factor of 50 to 2,000. In the case of whole sera with no detectable ECI, the inhibitory activity emerged following the HPLC sizing procedure (Fig. 4). This apparent activation or unmasking of ECI also occurred when serum was fractionated by anion exchange HPLC (Fig. 5 *A* and *B*). The activation of ECI by sizing or anion-exchange HPLC suggests that these separatory methods removed a component that stabilizes inactive ECI or suppresses the activity of ECI.

Alternatively, the conditions of sample handling and chromatography might have artifactually generated a substance with inhibitory functions in our biological assays. This possibility was tested in several different ways, and the following procedures all failed to activate ECI in normal sera: (*a*) dialysis or dilution of serum with medium or chromatography buffer, (*b*) exposure of serum to steel or silica components of HPLC columns without chromatographic separation, or (*c*) salting out of serum with ammonium sulfate (though the 50–70% saturated ammonium sulfate fraction contained material that could be activated by subsequent HPLC). It has also found that HPLC size fractionation of chromatography buffer, and of rabbit, mouse, or fetal calf sera did not generate ECI. Thus, ECI was not generated or activated by removal of electrolytes or other low molecular weight substances, by dilution, by
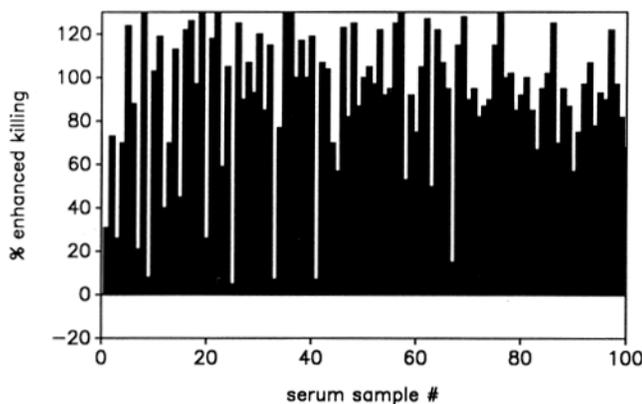
FIGURE 2. Population distribution of the eosinophil cytotoxic inhibitory activity in human sera. 100 anonymous serum samples (HIV and hepatitis B negative) were obtained from the Red Cross and tested at a concentration of 10% for the ability to inhibit the cytotoxic function of TNF-activated eosinophils.
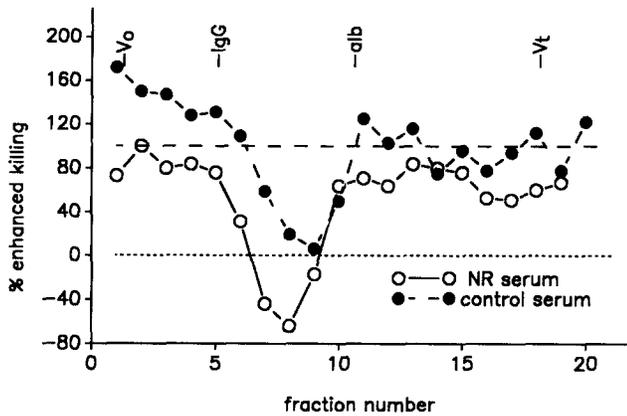
FIGURE 3. HPLC sizing analysis of the inhibitor. 100 μl of NR or normal serum was injected on a TSK 400 column, and 1 ml fractions were collected. The fractions were heat-inactivated (56°C for 30 min) and added to TNF-stimulated eosinophils at a concentration of 20%. The cytotoxic function of the eosinophils in the presence of 100 U/ml of TNF was determined at 36–40 h. The levels of killing in the presence (–––) or absence (···) of TNF are indicated. The elution times of human serum albumin and IgG are indicated.

exposure to dialysis membrane, steel, or silica, by some other property of the HPLC columns, or by nonspecific reactions involving molecules found in serum from other mammalian species.

We also considered an example of a nonspecific reaction that was described in another in vitro system (20, 21). It was demonstrated that low density lipoproteins, which may become oxidized during chromatography in aerated buffers (21), can suppress cytotoxic functions of human monocytes with respect to tumor cell targets. However, we found that high density, low density, and very low density lipoproteins did not suppress the cytotoxic function of TNF-stimulated eosinophils, either directly or after size exclusion HPLC (using de-gassed buffers according to our standard practice). The lipoprotein elution times did not overlap the elution time of ECI. However, size exclusion HPLC of lipoprotein-free serum generated ECI of 80,000–100,000 daltons. Therefore, the phenomenon of lipoprotein oxidation is not related to ECI.

We investigated the reversibility of the activation of ECI. When NR serum was mixed with normal sera in various proportions, the ECI titer was not diminished.
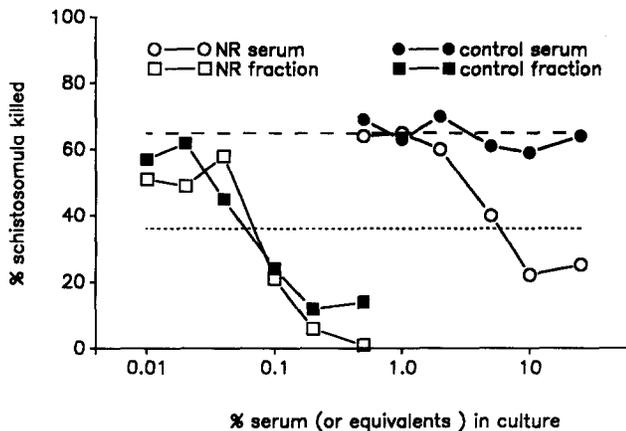

FIGURE 4. Increase in the titer of the inhibitor as a consequence of HPLC sizing chromatography. NR serum (same sample as Fig. 3), a normal serum sample with no inhibitory activity (same sample as Fig. 3), and respective pools of HPLC fractions 7–9 of these sera were tested for ECI activity at the indicated concentrations.
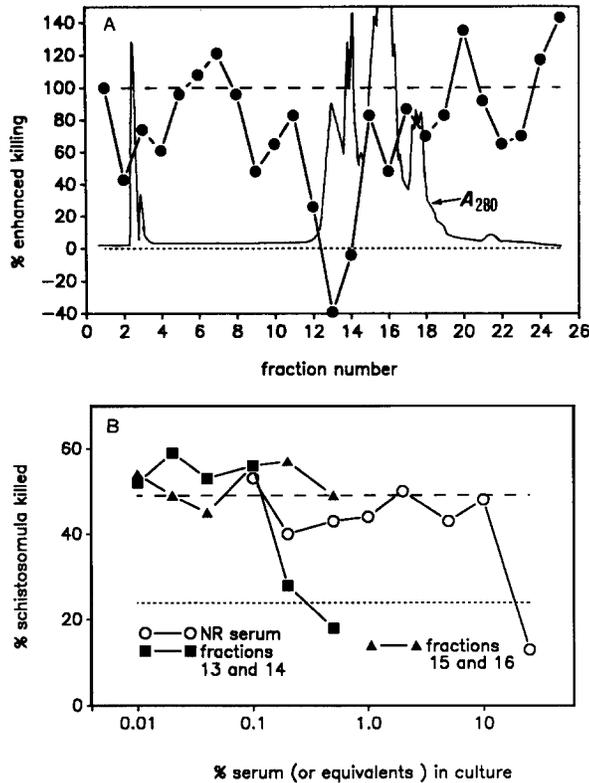
FIGURE 5. Increase in the titer of the inhibitor as a consequence of anion-exchange HPLC. (A) NR serum was injected on a DEAE-5PW column in low salt Tris buffer and eluted with a continuous gradient to Tris/0.5 M ammonium sulfate (see Materials and Methods). The elution profile is shown (relative absorbance at 280 nm). 2-ml fractions were collected and tested for ECI activity. Data are displayed as for Fig. 3. (B) ECI activity was titrated as in Fig. 4 for pooled fractions 13 and 14, pooled fractions 15 and 16, and the sample of NR serum from which these fractions were derived.

Similarly, when ECI prepared from NR or normal serum was mixed with the serum from which it was derived or with other HPLC fractions, the major part of ECI activity remained. Thus, the conversion to active ECI appeared to be irreversible.

ECI activity was reduced by heating at 80°C for 1 h or by treatment with trypsin, demonstrating that it is at least in part polypeptide (Fig. 6).

Although activated ECI was not generally detectable in normal sera until it had been fractionated (Fig. 4), the property of activatability and its apparent molecular weight suggested a possible relationship of ECI to factors of the contact activation pathway. To test this possibility, we obtained plasmas from patients deficient in factors XI, XII, prekallikrein, and high molecular weight kininogen. HPLC size fractionation of each sample generated normal titers of ECI. Thus ECI is neither identical to the contact activation factors, nor dependent on these factors for activation.

Preliminary Studies of the Mechanism of Action of ECI.   In the presence of antibody and TNF, eosinophils form aggregates, particularly on the surface of the target schistosomula. ECI, but not control preparations, reduced the formation of aggregates. To determine whether ECI could suppress eosinophil function in the absence of a living target organism, the effect of ECI on eosinophil adherence to plastic was tested. In a typical experiment, TNF increased eosinophil adherence by 367%, and an HPLC fraction containing ECI reduced TNF-enhanced adherence by 71%, with respect to a control HPLC fraction.
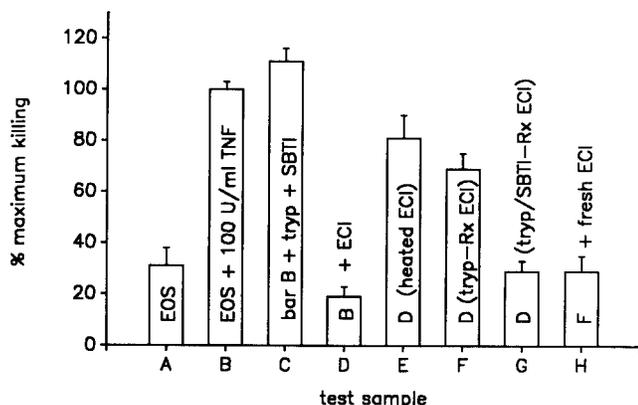
Figure 6. Sensitivity of ECI to heat or trypsin treatment. The results in three experiments with ECI (fractions 7-9 from Fig. 3) from NR serum and four experiments with ECI from different normal sera were essentially similar; in order to compare the results on the same scale, the level of killing in the presence of TNF with no inhibitors present was set equal to 100%. Values represent the mean percent maximum killing ± SE (n = 7). Killing was assayed under standard conditions when eosinophils were stimulated with: (A) control medium; (B) 100 U/ml of TNF; (C) 100 U/ml of TNF plus (1% trypsin [two treatments with 0.5% trypsin at 37°C for 30 min]) plus soybean trypsin inhibitor (fourfold excess neutralizing units); (D) TNF plus untreated ECI; (E) TNF plus treated ECI (treatment: 80°C for 1 h); (F) TNF plus treated ECI; (1 h trypsin, then SBTI); (G) TNF plus ECI (1 h SBTI and trypsin together); (H) sample F with fresh ECI added at end.

ECI did not have detectable toxic effects on eosinophils. The addition of ECI did not affect the uptake of trypan blue by eosinophils incubated in assay medium for periods up to 36 h or by nonadherent eosinophils removed from cytotoxicity cultures. Furthermore, incubation of eosinophils in medium with ECI for 1 or 12 h did not affect their ability to release H_2O_2, following stimulus with an optimal dose of phorbol myristate acetate. In one experiment, for example, PMA increased the rate of H_2O_2 production by a factor of 63.5 and 66.3, respectively, for eosinophils incubated 12 h with and without an optimal dose of ECI. By comparison, a 5-min preincubation with 0.01% sodium azide (azide removed by washing) limited the increase to a factor of 30.3. Thus, the effect of ECI appeared to be a form of specific regulation rather than a general toxicity.

The possibility was considered that ECI might have proteolytic activity, which might act by cleaving eosinophil surface molecules involved in adhesion. To test ECI for proteolytic activity, doses of ECI as high as 10 times the optimal concentration (in the assay of eosinophil cytotoxic function) were mixed with ^{125}I-labeled human serum albumin or egg albumin PBS or in medium containing 10% FCS (as for the eosinophil cytotoxicity assay). SDS gel electrophoresis detected significant degradation of the radiolabeled proteins by as little as 1 U/ml of trypsin in PBS, but no degradation with any dose of ECI. Further, treatment of ECI with the irreversible serine protease inhibitor diisopropyl fluorophosphate and dialysis in medium did not affect its ability to suppress eosinophil cytotoxic function. These experiments suggest that if ECI is a protease, it is of a different family and with a highly restricted specificity.

There are recent reports in the literature of factors from the urine of febrile patients that inhibit the toxic effects of TNF to tumor cell targets. Such substances have been purified and appear to act by binding to the TNF molecule and preventing the interaction between TNF and its receptor on the target cell surface (22-24). The

molecule known as uromodulin does not appear to inhibit the activity of TNF in standard tumor cell cytotoxicity assay, but its ability to bind to solid-phase TNF suggests a role in the modulation of TNF-mediated reactions (25). In view of this evidence, we wanted to learn whether ECI acts by interfering with the interaction of TNF and its receptor.

First, we considered whether the effects of ECI on eosinophils were specific for TNF. When 20 pM GM-CSF was used to stimulated eosinophil cytotoxic function, selected control sera (Nos. 51, 52, 53, and 54 from the Red Cross collection, see Fig. 2) (Table I) and various control HPLC sizing fractions (not shown) did not affect cytotoxic function. However, NR serum, chromatographically purified ECI from NR serum, and selected samples with detectable ECI (Nos. 25, 33, 41, and 67) all reduced the cytotoxic function of GM-CSF-stimulated eosinophils (Table I). Thus, the effect of ECI is not cytokine specific.

Next, ECI was tested for the ability to protect L929 cells from the toxic effects of TNF; it had no effect in the presence or absence of actinomycin D, when added to the culture after various times of incubation. In contrast, the TNF-specific mAb 245-10E11 protected the L929 cells when added during the first few hours of culture.

Finally, we examined the time dependence of the effects of ECI on eosinophil-mediated killing of schistosomula targets. The inhibitory effect of NR serum (not shown) or ECI (Fig. 7) could be observed when they were added to the eosinophils as late as 4 h after the TNF. mAb 245-10E11 also inhibited TNF-enhanced cytotoxic function, but only when it was added simultaneously with the TNF to the eosinophils (Fig. 7). In another set of experiments, ECI was added to the cultures at the beginning, but was removed by washing at various times. The effect of ECI on eosinophil cytotoxic function was observed when it was present for the first 2 h of culture. When

TABLE I

Inhibition of Cytotoxic Function of GM-CSF-stimulated Eosinophils by ECI

Inhibitor	Cytokine stimulation of eosinophils		
	None	100 U/ml TNF	20 pM GM-CSF
None	9*	48	100
10% NR serum	23	24	33
20% ECI‡	13	27	46
Red Cross serum no.§			
51	7	37	92
52	11	46	70
53	6	51	89
54	12	35	83
25	7	7	22
33	10	11	25
41	12	17	25
67	15	12	36

* The percent maximum killing of schistosomula (maximum killing = GM-CSF without inhibitor) assayed at 26–40 h, means of two experiments.

‡ Derived from NR serum, dilution factor relative to the serum = 50.

§ Samples 51–54 were selected for the absence of an inhibitory activity; samples 25, 33, 41, and 67 were selected for the presence of inhibitory activity (see Fig. 2).
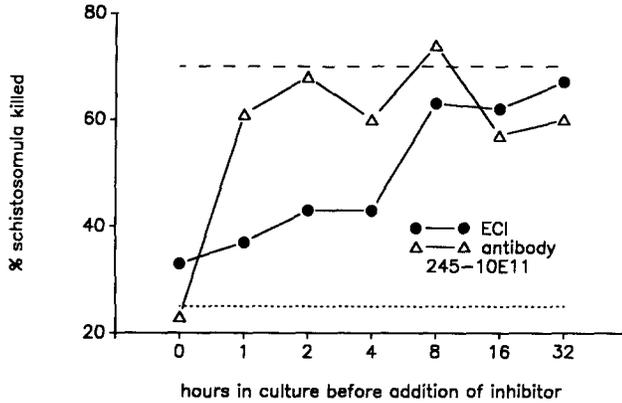
FIGURE 7. Time dependence of the effects of ECI and mAb 245-10E11 on the cytotoxic function of TNF-stimulated eosinophils. 20% ECI or 1,000 neutralizing units of mAb 245-10E11 was added to TNF-stimulated eosinophils at the indicated times of culture with schistosomula-specific antibody and schistosomula. The cultures were incubated further in order to assay of eosinophil cytotoxic function. The killing of schistosomula was scored at 36–40 h.

it was removed after shorter durations, the effects were diminished or undetectable (Fig. 8). Preincubation of eosinophils with ECI for up to 12 h at 37°C and removal of ECI by washing did not reduce the subsequent cytotoxic function of TNF-stimulated eosinophils. Thus, suppression of cytotoxic function required the presence of ECI for at least 2 h during the first 4–6 h of culture.

Taken together, the suppressive effect of ECI on GM-CSF-enhanced cytotoxic function, the failure of ECI to protect L929 cells from the toxic effects of TNF, and the nature of time dependence show that ECI does not act by binding to TNF or interfering with the initial interaction between TNF and the cell. ECI appears to act directly on eosinophil expression of cytotoxic function.

Ultrastructural study of the eosinophil cytotoxic reaction against schistosomula demonstrate that antibody-mediated discharge of eosinophil granule contents against the target surface begins within 2 min of contact and continues for more than 12 h. The target organisms begin to die after 8–12 h of culture, presumably as a result of cumulative damage to the tegument by toxic secretions (26). In this context, our results suggest that ECI acts on an event early in the adherent and degranulation functions of eosinophils against the targets.

In summary, we identified a human subject with a history of severe allergic dermatitis, whose eosinophils and neutrophils frequently failed to respond to TNF in
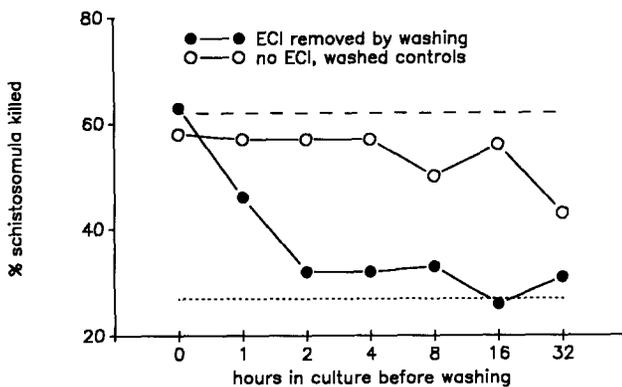

FIGURE 8. The duration of exposure to ECI required for the inhibitory effect. TNF-stimulated eosinophils were incubated with antibody, schistosomula, and either ECI or control buffer for the indicated time before washing. Then the individual culture contents were diluted by a factor of >100 in medium, centrifuged, and re-cultured in a volume of 250 µl with fresh antibody and TNF. The killing of schistosomula was scored at 36–40 hours.

in vitro assays. Sera from this subject, from two patients with severe hypereosinophilia, and from 4 of 100 anonymous donors contained a substance (ECI) that suppressed the cytotoxic function of cytokine stimulated eosinophils. ECI is a polypeptide of 80–100 kDa. ECI or an inactive precursor of ECI was present in all sera that were tested. Fractionation of sera increased irreversibly the activity of ECI. Collectively, the association of serum ECI with inflammation and the in vitro activities of ECI suggest that this substance is part of a natural feedback mechanism to suppress functions of cytokine-stimulated eosinophils in inflammation.

Summary

A human subject (NR) was identified whose eosinophils and neutrophils failed to respond to TNF in vitro in 29 of 33 experiments, using several biological assays. There was a response rate to TNF of 100% among 37 control subjects whose leukocytes were tested in parallel. NR serum contained an activity that inhibited the cytotoxic function of TNF- and GM-CSF-stimulated normal human eosinophils. A similar activity was detected in 4 of 122 control sera and in sera of two subjects with hypereosinophilia. This activity (ECI) had an apparent molecular weight of 80,000–100,000 and was sensitive to heating at 80°C or to trypsin treatment. HPLC sizing chromatography increased the titer of ECI by a factor of 50 to 2,000 in experiments using NR serum or other sera with detectable inhibitory activity. In seven experiments using sera with no inhibitory activity, HPLC generated ECI of the same apparent molecular weight. The effect of HPLC on ECI activity required the separation of serum components and did not result from exposure to HPLC system components or other sample processing methods. This suggests that ECI in serum can be stabilized in an inactive or partially active form and that HPLC removes the stabilizing component. ECI suppressed TNF-stimulated eosinophil cytotoxic function when added to cultures up to 4 h after exposure of eosinophils to cytokine. However, ECI did not protect L929 cells from the toxic effects of TNF. Thus, ECI did not act by preventing the initial interaction of TNF with eosinophils or by interfering with the binding of TNF to its receptor on L929 cells. The results suggest that ECI is a component of a feedback mechanism that suppresses functions of cytokine-activated eosinophils in inflammation.

References

1. Silberstein, D. S., and J. R. David. 1986. Tumor necrosis factor enhances eosinophil toxicity to *Schistosoma mansoni* larvae. *Proc. Natl. Acad. Sci. USA.* 83:1055.
2. Lamas, A. M., C. M. Mulroney, and R. P. Schleimer. 1988. Studies on the adhesive interaction between purified human eosinophils and cultured vascular endothelial cells. *J. Immunol.* 40:1500.
3. Shalaby, M. R., B. B. Aggarwal, E. Rinderknecht, L. P. Svedersky, B. S. Finkle, and M. A. Palladino. 1985. Activation of human polymorphonuclear neutrophils by interferon-γ and tumor necrosis factors. *J. Immunol.* 135:2069.
4. Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas. 1985. Stimulation of adherence of neutrophils to umbilical vein epithelium by human recombinant tumor

necrosis factor. *Proc. Natl. Acad. Sci. USA.* 82:8667.

5. Klebanoff, S. J., M. A. Vadas, J. M. Harlan, L. H. Sparks, J. R. Gamble, J. M. Agosti, and A. M. Waltersdorph. 1986. Stimulation of neutrophils by tumor necrosis factor. *J. Immunol.* 136:4220.

6. Larrick, J. W., D. Graham, K. Toy, L. S. Lin, G. Senyk, and B. M. Fendly. 1987. Recombinant tumor necrosis factor causes activation of human granulocytes. *Blood.* 69:640.

7. Perussia, B., M. Kobayashi, M. E. Rossi, I. Anegon, and G. Trinchieri. 1987. Immune interferon enhances functional properties of human granulocytes: Role of Fc receptors and effect of lymphotoxin, tumor necrosis factor, and granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 138:765.

8. Philip, R., and L. B. Epstein. 1986. Tumor necrosis factor as an immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon, and interleukin-1. *Nature (Lond.).* 323:86.

9. Bevilaqua, M. P., J. S. Pober, G. R. Majeau, W. Fiers, R. S. Cotran, and M. A. Gimbrone. 1986. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proc. Natl. Acad. Sci. USA.* 83:4533.

10. Lapierre, L. A., W. Fiers, and J. S. Pober. 1988. Three distinct classes of regulatory cytokines control endothelial cell MHC expression. Interactions with immune γ-interferon differentiate the effects of tumor necrosis factor and lymphotoxin from those of leukocyte α-interferon and fibroblast β-interferon. *J. Exp. Med.* 167:794.

11. Sugarman, B. J., B. B. Aggarwal, P. E. Hass, I. S. Figari, M. A. Palladino, and H. M. Shepard. 1985. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and transformed cells in vitro. *Science (Wash. DC).* 230:943.

12. Dayer, J.-M., B. Beutler, and A. Cerami. 1985. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. *J. Exp. Med.* 162:2163.

13. Wang, A. M., A. A. Creasey, M. B. Ladner, L. S. Lin, J. Strickler, J. N. Van Arsdell, R. Yamamoto, and D. F. Mark. 1985. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science (Wash. DC).* 228:149.

14. Fendly, B. M., K. J. Koy, A. A. Creasey, C. R. Vitt, J. W. Larrick, R. Yamamoto, and L. S. Lin. 1987. Murine monoclonal antibodies defining neutralizing and non-neutralizing epitopes or recombinant tumor necrosis factor. *Hybridoma.* 6:359.

15. Silberstein, D. S., W. F. Owen, J. C. Gasson, J. F. DiPersio, D. W. Golde, J. C. Bina, R. Soberman, K. F. Austen, and J. R. David. 1986. Enhancement of human eosinophil cytotoxicity and leukotriene synthesis by biosynthetic (recombinant) granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 137:3290.

16. Vadas, M. A., J. R. David, A. E. Butterworth, N. T. Pisani, and T. Siongok. 1979. A new method for the purification of human eosinophils and neutrophils and a comparison of the ability of these cells to damage schistosomula of *Schistosoma mansoni. J. Immunol.* 122:1228.

17. Boyum, A. 1968. Ficoll-hypaque method for separating mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77.

18. Root, R. K., J. Metcalf, N. Oshino, and B. Chance. 1975. H2O2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. *J. Clin. Invest.* 55:945.

19. Denizot, F., and R. Lang. 1986. Rapid colorimetric assay for cell growth and survival. *J. Immunol. Methods.* 89:271.

20. Jurgens, G., H. F. Hoff, G. M. Chisholm, and H. Esterbauer. 1987. Modification of human serum low density lipoproteins by oxidation — characterization and pathophysiological implications. *Chem. Phys. Lipids.* 45:315.

21. Justement, L. B., S. T. Patel, H. A. I. Newman, and B. S. Zwilling. 1984. Inhibition of macrophage-mediated tumor cell destruction by oxidized lipoproteins. *J. Natl. Cancer Inst.* 73:469.

22. Seckinger, P., S. Isaaz, and J.-M. Dayer. 1988. A human inhibitor of tumor necrosis factor-α. *J. Exp. Med.* 167:1511.

23. Seckinger, P., S. Isaaz, and J.-M. Dayer. 1989. Purification and biologic characterization of a specific tumor necrosis factor α inhibitor. *J. Biol. Chem.* 264:11966.

24. Engelmann, H., D. Aderka, M. Rubinstein, D. Rotman, and D. Wallach. 1989. A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity. *J. Biol. Chem.* 264:11974.

25. Sherblom, A., P., J. M. Decker, and A. V. Muchmore. 1988. The lectin-like interaction between recombinant tumor necrosis factor and uromodulin. *J. Biol. Chem.* 263:5418.

26. Caulfield, J. P., H. L. Lenzi, P. P. Elsas, and A. J. Dessein. 1985. Ultrastructure of the attack of eosinophils stimulated by blood mononuclear cell products on schistosomula of *Schistosoma mansoni*. *Am. J. Pathol.* 120:380.