TUMOR NECROSIS FACTOR α/CACHECTIN STIMULATES EOSINOPHIL OXIDANT PRODUCTION AND TOXICITY TOWARDS HUMAN ENDOTHELIUM

By A. SLUNGAARD, G. M. VERCELLOTTI, G. WALKER, R. D. NELSON,* AND H. S. JACOB

*From the Departments of Internal Medicine and *Surgery, University of Minnesota, Minneapolis, Minnesota 55455*

Macrophages exposed to endotoxin secrete copious amounts of tumor necrosis factor α/cachectin (TNF), a potent proinflammatory cytokine that stimulates polymorphonuclear leukocyte (PMN)[1] adhesion to endothelium (1), phagocytosis, degranulation, and oxidant generation (2, 3), as well as endothelial cell procoagulant activity (4). Though it normally functions to help the host rid itself of pathogenic organisms (5), TNF also has the potential, as do other proinflammatory mediators, to cause phagocyte damage to host cells. Especially vulnerable in this regard are host endothelial cells, interposed as they are between activated phagocytes and extravascular tissue. For example, large intravenous infusions of purified recombinant human TNF (rhTNF) reproduce all the cardinal features of endotoxin-induced shock in rats: rapid death from hypotension caused by a diffuse capillary leak syndrome manifesting as hemoconcentration, tissue edema, and histopathologic evidence of PMN margination, agglutination, and extravasation (6). On the other hand, mice whose macrophages are incapable of producing TNF in response to endotoxin are also resistant to endotoxin-induced lethal shock (7). Thus, in pathologic circumstances, TNF can provoke a deleterious, even lethal interaction between PMNs, endothelium, and the coagulation cascade.

Eosinophils (EOs) participate, as do PMNs, in a variety of pathologic inflammatory states reflecting endothelial cell damage, such as cutaneous and systemic vasculitis, pneumonitis, and eosinophilic endocarditis (8). Particularly striking is the unusual endothelial cell injury that can complicate chronic hypereosinophilic states, irrespective of their cause. For unclear reasons, the endothelial lining of the heart, the endocardium, is prominently involved, promoting thrombus formation with embolic sequelae, valvular dysfunction, and mechanical impairment that culminates

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Address correspondence to Arne Slungaard, P. O. Box 480 UMHC, University of Minnesota, Minneapolis, MN 55455.

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; EBSS, Earle's buffered salt solution; ECP, eosinophil cationic protein; EOs, eosinophils; EPO, EO peroxidase; HUVEC, human umbilical vein endothelial cell; MBP, major basic protein; PAF, platelet-activating factor; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase.

in florid congestive heart failure (9, 10). Hypereosinophilic heart disease, though rare in Western societies, is estimated to cause 10–20% of cardiac deaths in tropical Africa (11), where chronic hypereosinophilia (presumably caused by filariasis and other parasitic infestations) is endemic (12). Individuals with hypereosinophilic heart disease have peripheral blood EOs that show morphologic and metabolic evidence of activation (13), as well as high serum levels of potentially cytotoxic eosinophil-specific granule constituents, such as major basic protein (MBP) (14), suggesting that endothelial damage results from chronic activation and misapplication of the potent EO cytotoxic armamentarium. The factor(s) responsible for this activation are, however, obscure.

TNF is potentially such a factor because it has been shown to stimulate two important aspects of EO function: adhesion to human endothelium (15) and antibody-dependent cellular cytotoxicity (ADCC), assayed as killing of antibody-opsonized schistosomula (16). Moreover, abnormally elevated TNF levels are detectable in the serum of humans with chronic parasitic infestations (17), a setting in which chronic eosinophilia commonly occurs. We hypothesized that such "pathologically" elevated concentrations of TNF might provoke EO toxicity towards unopsonized endothelial cells, and so promote tissue injury in hypereosinophilic states. We find that concentrations of TNF attainable in human disease states stimulate highly homogeneous populations of human EOs to damage human umbilical vein endothelial cell (HUVEC) monolayers, a model of human endothelium. TNF directly influences both endothelium and EOs, rendering HUVEC more susceptible to destruction by activated EOs and reagent H_2O_2, and activating EO generation of cytotoxic oxygen species. These findings suggest that abnormally elevated serum levels of TNF may play a role in the pathogenesis of tissue damage in hypereosinophilic states, in part by simultaneously activating EO oxidant generation while diminishing endothelial antioxidant defenses.

Materials and Methods

Recombinant *Escherichia coli*–derived human TNF (rhTNF) was generously supplied by the Cetus Corp. (Emeryville, CA.) The TNF preparation had a specific activity as assessed by L929 assay of 2.4×10^7 U/mg protein, and was >98% pure by SDS-PAGE analysis. The endotoxin contamination of rhTNF, as assayed by the limulus amoebocyte lysate assay, was 3 pg/1,000,000 U. Therefore, at the concentrations used in the course of these studies (<1,000 U/ml) the endotoxin contribution from the TNF preparation was well below the 1–10 ng/ml required for priming of the PMN respiratory burst (18). TNF was heat inactivated by heating to 90°C. for 15 min (19). Rabbit anti-TNF serum, also supplied by Cetus Corp., was produced by repeated injections of rabbits with rhTNF; a 1:100 dilution of the resulting antiserum neutralized 4,000 U/ml TNF activity in an equal volume. Control serum was obtained from an unimmunized rabbit. The tetrapeptide FMLP, superoxide dismutase (SOD), catalase, Na fluoresceinate, and hydrogen peroxide (30% solution) were obtained from Sigma Chemical Co. (St. Louis, MO). Percoll was obtained from Pharmacia Fine Chemicals (Piscataway, NJ), PMA from Consolidated Midland Corp. (Brewster, NY), Earle's buffered salt solution (EBSS) and HBSS from Gibco Laboratories (Grand Island, NY), FITC mAb Mol (IgM) from Coulter Immunology (Hialeah, FL), ^{51}Cr from Amersham Corp. (Arlington Heights, IL), methylprednisolone from Upjohn (Kalamazoo, MI), heparin from Elkins-Sinn, Inc. (Cherry Hill, NJ), Hetastarch (6% hydroxyethyl starch in normal saline) from DuPont Co. (Wilmington, DE), and 24-well plates (2 cm^2/well) from Costar (Cambridge, MA). The platelet-activating factor inhibitor BN52021 was kindly supplied by Dr. Pierre Braquet (Paris, France).

Eosinophil Purification. EOs were isolated from peripheral blood (50–200 ml) of individuals with mild to moderate peripheral blood eosinophilia (4–39%) with the following diagnoses: primary hypereosinophilia (1), allergy (6), Herpes gestationis (1), pulmonary aspergillosis (3), filariasis (2), graft-vs.-host disease (1), asthma (2), and lymphoma (1). Isolation of EOs was accomplished using a modification of the FMLP-EDTA technique previously described by Roberts and Gallin (20). Peripheral blood was obtained by venipuncture and was drawn directly into 60-cc syringes containing 20 cc of Hetastarch and heparin (10 U/ml). After sedimenting 30 min at room temperature, the red cell–poor supernatant plasma was collected and pelleted at 1,000 *g* for 5 minutes. The resulting leukocyte-free plasma was decanted and saved, while contaminating red cells in the granulocyte pellet were removed by hypotonic lysis followed by two washes in PBS + 0.4% Na citrate (PBS/citrate). Granulocytes were then resuspended in the leukocyte-free plasma, retained from the previous step, to which EDTA (0.3% final concentration) had been added. FMLP was added to a final concentration of 10^{-6} M, and the cells were incubated 15 min at 37°C in a rocking water bath. Discontinuous Percoll gradients were prepared by diluting iso-osmolar stock Percoll (d = 1.130, obtained by diluting Percoll with 10× concentrated PBS, adjusting osmolality to 310 mOsm), then further diluting with PBS/citrate to obtain solutions with densities of 1.095 and 1.080. 5 ml of the 1.080 solution was layered atop 2 ml of d = 1.095 solution in a 15-ml conical tube (2095; Falcon Labware, Oxnard, CA). Finally, 5-ml granulocytes suspended in autologous plasma/EDTA were layered over the d = 1.080 Percoll. Tubes were then spun at 400 *g* at room temperature for 25 min in a swinging bucket rotor. Cells were then harvested from the interface between the two different Percoll densities, diluted with PBS/citrate, pelleted, and washed two times in PBS/citrate. Aliquots of the resulting suspension were used to prepare cytospins, which were subsequently stained with Wright's stain in order to assess EO purity. The cell yield using this technique was 60–90% of that present in the sedimented plasma. EO purity was 85–99.5% (mean 94%); contaminating cells were largely PMNs.

Preparation of HUVEC Monolayers. HUVEC were prepared by collagenase treatment of umbilical cords as previously described (21). Cells were seeded in 24-well plates (Costar) (2 cm^2 per well) and utilized as primary cultures upon achieving confluence, usually 5–7 d after seeding.

^{51}Cr Release Cytotoxicity Assay. Culture medium was aspirated from confluent endothelial cell monolayers, which were then washed two times in 1 ml of HBSS (37°C). 200 μl of RPMI medium containing 1–2 μCi of ^{51}Cr was layered atop each monolayer, then incubated 2 h at 37°C in a 5% CO_2 atmosphere. Tissue culture medium was then aspirated and the cells washed three times in 1 ml of HBSS, 37°C. After the final wash the labeled monolayers were overlaid with EBSS containing 5 × 10^6 EOs and any other reagents in a final volume of 1 ml, yielding an effector (EO) to target (endothelial cell) ratio of 20–25:1. EO contact with the endothelial cell monolayers was ensured by gentle (50 *g* for 4 min) centrifugation of the 24-well plates. Inhibitors, such as heparin and methylprednisolone, were added before centrifugation, and agonists, such as TNF and PMA, after centrifugation. Plates were incubated overnight 16 h at 37°C in a 5% CO_2 incubator, then centrifuged (1,000 *g* for 10 min) to pellet any endothelial cells that may have detached but not lysed. The supernatant fluid was carefully aspirated and 1 ml of 1.0 M NaOH added to solubilize counts in the adherent and pelleted cells. Supernatant and pellet fractions were counted in a gamma counter. Cytotoxicity was expressed as percent specific ^{51}Cr release, calculated as follows: percent specific ^{51}Cr release = $100 \times [(A-C)/(B-C)]$, where A = cpm in the supernatant of any given well; B = total counts (pellet + supernatant) in that well; and C = the mean cpm "spontaneous" release of ^{51}Cr into supernatant buffer of four wells containing labeled monolayers and EBSS only. Typically, spontaneous release was 15–20% of the total counts/well. All experiments were conducted using triplicate or quadruplicate determinations of each treatment group.

Assay of Superoxide Anion and HOBr Production. Superoxide anion production was measured as SOD-inhibitable reduction of cytochrome *c* (22) from EOs adhering to the bottoms of serum-coated tissue culture plastic wells. 200 μl of FCS was aliquoted into each well of a 24-well, 2-cm^2 tissue culture plate. After 0.5 h at 37°C, the serum was aspirated and each well washed twice with 1 ml of 37°C HBSS. 10^6 EOs in 1 ml of EBSS containing 75 μM cytochrome *c* was then aliquoted into each well. Contact with the serum-coated plastic well

bottoms was promoted by gentle (50 *g* for 4 min) centrifugation, after which SOD (20 μg/ml) and agonists, such as TNF or PMA, were added. Wells were incubated overnight at 37°C in a 5% CO_2 atmosphere, after which wells were spun at 1,000 *g* for 10 min to pellet loose cells, the supernatant fluid was aspirated, and OD_{550} was measured before and after addition of a few granules of KFeCN.

HOBr was measured in similar preparations of 10^6 EOs attached to serum-coated 2-cm^2 tissue culture wells by conversion of Na fluoresceinate to eosin yellow (23, 24). Each well contained a final volume of 1 ml EBSS supplemented with 32 μM sodium fluoresceinate and 1 mM sodium bromide. HOBr production was assayed by pelleting unattached cells (1,000 *g* for 10 min) and determining supernatant fluid OD_{519}. The OD_{519} of buffer from control wells containing all the reagents but no EO was subtracted from each value, and a measured micromolar extinction coefficient of 0.078 was used to determine molarity of eosin yellow and, therefore, HOBr production.

FACS Analysis of CR3 ($CD11_b$) Expression. 200,000 cell aliquots of EOs (prepared as above) or PMNs (prepared by centrifugation of buffy coats over Percoll; d = 1.075 [25]) were suspended in 100 μl PBS-0.2% BSA-0.1% sodium azide (PBS-BSA-SA) and incubated 15 min at 37°C before adding agonists (100 U/ml TNF, 20 ng/ml PMA, or 10^{-6} M FMLP), and incubating another 30 min at 37°C. 10 μl of FITC-Mol stock solution (5 μg protein) was then added to the cells by gentle vortexing before incubating 30 min on ice in the dark. Labeled cells were washed three times in PBS-BSA-SA, suspended in 500 μl PBS-1% paraformaldehyde, and stored in the dark at 4°C until analyzed by flow cytometry 2–18 h later. Flow cytometric analysis of stained leukocytes was accomplished on a Cytofluorograf 50H with a 2150 computer (Ortho Diagnostic Systems Inc., Westwood, MA) equipped with an argon (488, 250 mW) and a helium-neon (632.8 nm, 0.8 mW) laser. Gain was monitored daily with the use of fluorescein-stained calf thymocytes (Fluorotrol; Ortho Diagnostic Systems, Inc.) and adjusted to give a mean channel fluorescence value of 155. Fluorescence of stained cells was quantified by assaying right angle green fluorescence >530 nm on a linear scale. For each analysis, 10,000 cells were examined; a common setting was used for PMNs and EOs.

Pretreatment of Eosinophils or HUVEC with TNF. In one of studies, either HUVEC or EOs were exposed for 4 h to 100 U/ml TNF in isolation before washing away unbound TNF, then recombining EOs and target cells. HUVEC were exposed to TNF in tissue culture medium containing 100 U/ml of TNF for 4 h at 37°C under 5% CO_2 atmosphere, followed first by aspiration of medium and two washes in 37°C HBSS, then by addition of 5×10^6 untreated EOs/well. Alternatively, EOs were suspended (2.5×10^6 cells/ml) in EBSS containing 100 U TNF/ml, and incubated 4 h at 37°C in a 5% CO_2 atmosphere with gentle agitation. EOs were then pelleted and washed two times in large volumes of EBSS before being resuspended in EBSS and 5×10^6 EOs layered over each untreated endothelial cell monolayer. Separate experiments assessed the effects of long-term (18-h) exposure of endothelial cells to TNF upon their subsequent vulnerability to killing by activated EOs and H_2O_2. For these experiments, tissue culture medium was aspirated from all 24 wells of a 24-well plate, then half the wells were replaced with fresh tissue culture medium and the other half with fresh tissue culture medium containing 100 U/ml TNF. The plates were incubated overnight at 37°C under a 5% CO_2 atmosphere. Wells were then aspirated, washed, and labeled with ^{51}Cr, as described above, before the addition of 1 ml EBSS or EBSS containing either reagent H_2O_2 or EOs (5×10^6/well) with or without PMA (10 ng/ml).

Statistics. A student's t test was used to assess the significance of differences between experimental groups.

Results

As shown in Fig. 1, addition of rhTNF stimulates a dose-dependent increase in endothelial cell ^{51}Cr release caused by EOs from two representative donors (one with low, the other with high unstimulated [i.e., in the absence of any known stimulus] toxicity for HUVEC). Although the shapes of the dose-response curves differ, in both cases, 100 U/ml TNF (a level within the range measurable in serum of patients

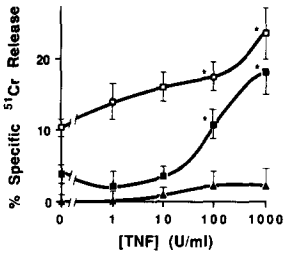

FIGURE 1. Dose-dependent stimulation of EO damage to HUVEC by TNF. 2-cm^2 monolayers of ^{51}Cr-labeled HUVEC were overlaid with 1 ml EBSS containing the indicated concentrations of TNF with no further additions (▲) or with 5 × 10^6 EOs from two different donors (□ and ■). After 16 h of incubation, percent specific ^{51}Cr release was calculated as described in Materials and Methods. An asterisk indicates cytotoxicity significantly greater ($p < 0.05$) than that in the absence of TNF. Error bars ± SEM.

with neoplastic diseases and parasitic infestations [17]) significantly increases EO toxicity for endothelium. 1,000 U/ml TNF further stimulates EO toxicity for HUVEC without causing significant direct toxicity for HUVEC. However, since this latter TNF dose is outside the range measured in human disease states, the remainder of our cytotoxicity studies were conducted using 100 U/ml rhTNF. Nevertheless, direct toxicity of TNF for endothelium at these concentrations is minimal and becomes significant only at levels of 10,000 U/ml or more (not shown).

Pooled results from 16 separate experiments assaying the effect of 100 U/ml TNF upon EO toxicity for HUVEC are presented in Table I. In aggregate, TNF, which at this dose does not itself injure endothelium, stimulates EO cytolysis of endothelium nearly 2.5-fold at the high (20–25:1) E/T ratio used. TNF-stimulated EO damage to endothelium at an 8:1 E/T ratio was 67% of that at 25:1 (not shown). Because of the large variability of both unstimulated (10.8 ± 12.1% SD specific ^{51}Cr release) and TNF-stimulated (21.4 ± 15.6%) EO toxicity in the pooled experiments, and the contrastingly tight correlation ($r = 0.95$) between unstimulated and TNF-stimulated EO toxicity for each individual experiment, the cytotoxicity results have been normalized and are expressed as percent that of unstimulated EO for each endothelial cell plate. To put this magnitude of TNF-stimulated EO toxicity in per-

TABLE I
Effect of TNF on EO Toxicity for HUVEC

Eosinophil preparation	Specific ^{51}CR release as percent that of EOS alone	p Value of difference
	% ± *SEM*	
EOs (5 × 10^6/well)	100 ± 25	-
EOs and TNF (100 U/ml)	243 ± 20	< 0.001*
EOs + anti-TNF serum (1:1,000)	50 ± 19	< 0.001‡
EOs + control serum (1:1,000)	185 ± 31	NS‡
EOs + polymyxin B (1 ng/ml)	245 ± 45	NS‡

^{51}Cr-labeled monolayers of HUVEC were overlaid with 1 ml EBSS containing 5 × 10^6 EOs, EOs with 100 U/ml TNF (EOs + TNF), or EOs + TNF with the indicated reagents. Specific ^{51}Cr release caused by EOs alone = 100% for each endothelial cell plate. 100 U/ml TNF alone caused ^{51}Cr release 27 ± 10% that of EOs.

* vs. EOs.

‡ vs. EOs + TNF.

spective, we find that EOs (n = 6) maximally stimulated with 100 ng/ml PMA cause 36 ± 12% specific ^{51}Cr release. The increased toxicity of EOs towards endothelium in the presence of TNF does not represent a direct toxic effect of TNF upon EOs with consequent release of cytotoxic EO granule constituents, because EOs recovered from endothelial cell monolayers after overnight incubation in the presence or absence of TNF both retain 90–95% viability, as assessed by trypan blue dye exclusion, and show no gross evidence of disruption or degranulation as assessed in Wright-stained cytospin specimens (not shown). Of note, EOs incubated overnight in EBSS without an endothelial cell monolayer were only 50% viable, thus confirming the previous observation by Rothenberg et al. (26) that coculture of eosinophils with endothelial cells greatly increases their longevity.

That TNF, and not some other contaminant, such as endotoxin, in our TNF preparation was responsible for stimulation of EO toxicity is also shown in Table I. Serum from a rabbit immunized against rhTNF significantly attenuates TNF-stimulated EO toxicity for HUVEC, whereas control rabbit serum does not. Moreover, 1 ng/ml polymyxin B, which stoichiometrically inactivates endotoxin (<10 fg/1,000 U in our TNF preparation), did not affect TNF/EO toxicity for HUVEC.

To examine mechanisms responsible for TNF-stimulated EO toxicity for endothelium, we assayed the effects of five potential modulators of EO function: (*a*) heparin, a polyanionic glycosaminoglycan known to block the killing of *Trypanosoma cruzi* blood stream forms both by EOs (27) and their cytotoxic cationic granule constituent, MBP (28); (*b*) methylprednisolone, a water-soluble glucocorticoid with protean antiinflammatory effects, including inhibition of EO chemotaxis (29), blockade of EO degranulation (30), and, as previously shown by us, parallel attenuation of C5a-induced EO superoxide production and cytotoxicity for HUVEC (31); (*c*) superoxide dismutase and catalase, enzymes that act sequentially to catalyze first the dismutation of superoxide anion to H_2O_2, then the two electron reductions of H_2O_2 to H_2O; (*d*) BN52021, a potent inhibitor of the effects of platelet-activating factor (PAF), an acether phospholipid metabolite produced by a variety of cells, including both EOs (32) and TNF-exposed endothelium (33), which is a potent agonist of EO respiratory burst activation (34), leukotriene production (34), and degranulation (35); and (*e*) 100 μM Br^-, a physiologically relevant concentration of this halide (not normally present in EBSS) which is preferentially oxidized by EO peroxidase (EPO) but not by PMN peroxidase (MPO) (36). We have shown (37) that the resulting HOBr is extremely toxic for several types of endothelium, including HUVEC, whereas Br^- itself is completely innocuous. Thus, augmentation of EO toxicity by Br^- implicates EPO participation in killing mechanisms. As shown in Fig. 2, both heparin (10 U/ml) and methylprednisolone (10 μg/ml) significantly attenuate TNF stimulation of EO toxicity for endothelium, whereas SOD/catalase and BN52021 are without discernible effect. In contrast, addition of 100 μM Br^- significantly enhances TNF-stimulated EO killing of endothelium.

Since TNF has been shown to affect directly both endothelium and PMNs, we next determined whether TNF-promoted EO toxicity for HUVEC was due to isolated effects of TNF solely upon EOs or, alternatively, endothelial target cells. Previous work has shown that isolated pre-exposure of either PMNs or HUVEC to TNF before their being combined enhances their subsequent adhesion to each other (1). Similarly, pre-exposure of PMNs to TNF followed by washing enhances their subse-

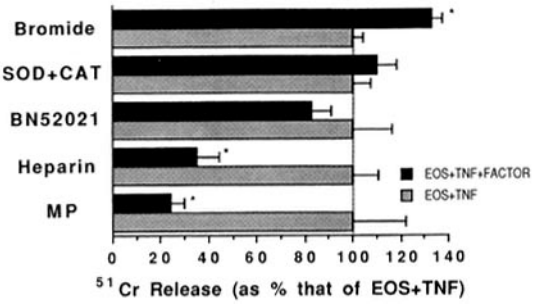

FIGURE 2. The effect of five modulators of EO cytotoxicity mechanisms upon TNF-dependent EO toxicity for HUVEC. 2-cm^2 monolayers of ^{51}Cr-labeled HUVEC were incubated 16 h in the presence of 5 × 10^6 EOs and 100 U/ml TNF with no further additions (*stippled bars*), or further supplemented with the following factors (*black bars*): 100 μM NaBr (Bromide); 100 μg/ml superoxide dismutase and 100 μg/ml catalase (SOD+CAT); 200 μM PAF inhibitor BN52021 (BN52021); 10 U/ml heparin (Heparin); or 10 μg/ml methylprednisolone (MP) before determining specific ^{51}Cr release. Mean specific ^{51}Cr release of EOS+ TNF = 100% for each endothelial plate used (n = 3–9 for each factor studied). An asterisk indicates cytotoxicity significantly different ($p < 0.05$) from that of EO+TNF. Error bars ± SEM.

quent lysis of HUVEC (38) and tumor cells (39). Accordingly, we exposed either EOs or HUVEC in isolation to 100 U/ml TNF for 4 h, after which unbound TNF was removed by thorough washing. EOs were then layered over HUVEC monolayers and incubated 16 h. As shown in the two bars on the right in Fig. 3, preexposure of either EOs or HUVEC in isolation for 4 h before their being recombined fails to reproduce the TNF-dependent stimulation of HUVEC killing seen when TNF was present along with EO for the full 16 h of coincubation (Fig. 3, *second bar from left*). Thus, in contrast to PMNs (38), EOs are not irreversibly activated to kill unopsonized endothelium by prior exposure to TNF.

TNF directly activates an important cytotoxic mechanism of EOs, the respiratory burst, but only when they adhere to "physiologic" surfaces. In studies not shown, we found that 100 U/ml of TNF caused scanty NBT reduction in a small (~12%) proportion of EOs in stirred suspensions in HBSS. However, the same concentration of TNF failed to stimulate a reproducible increase in superoxide anion production, assayed as cytochrome *c* reduction, in stirred suspensions of EOs. Because Nathan (40) has shown that PMNs must first adhere to a "physiologic" surface (e.g., serum- or fibronectin-coated plastic; endothelial cell monolayers) for TNF to stimulate PMN superoxide anion production, we studied EOs adhered to the bottom of

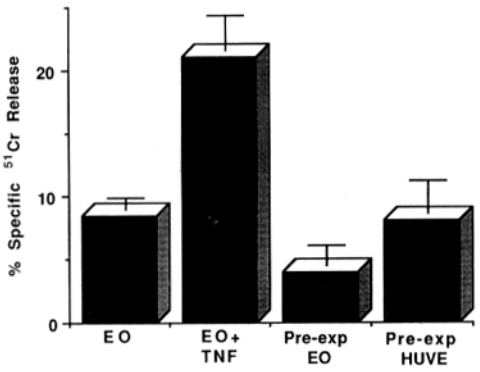

FIGURE 3. Effect of isolated pre-exposure of either EOs or HUVEC to TNF upon subsequent EO-dependent endothelial cell cytotoxicity. (EO) 5 × 10^6 EOs/2-cm^2 HUVEC monolayer, 16-h incubation; (EO+TNF) EOS + 100 U/ml TNF, 16 h incubation; (Pre-exp EO) EOs exposed 4 h to 100 U/ml TNF, then thoroughly washed before being layered atop untreated HUVEC monolayers, 16-h incubation thereafter; (Pre-exp HUVEC) HUVEC monolayers exposed 4 h to 100 U/ml TNF, thoroughly washed, then overlaid with untreated EOS, 16-h incubation thereafter. Error bars ± SEM.

FCS-coated wells. Under these conditions, addition of 100 U/ml of TNF, but not heat-inactivated TNF, consistently stimulates a twofold increase of EO superoxide anion production (Table II). Indeed, TNF-stimulated EOs produce nearly half as much superoxide anion as EOs maximally stimulated by 100 ng/ml PMA. TNF stimulates a low-grade, long-term activation of the EO respiratory burst, because at 4 h, superoxide anion production was only half that measurable after overnight incubation (not shown). In parallel studies, 100 U/ml TNF more than doubles EO production of HOBr, an oxidant generated by EO peroxidase (EPO) from H_2O_2 that is far more potently cytolytic for human endothelium than H_2O_2 (37). For perspective, 10^6 TNF-stimulated EOs produce ~3 nmol HOBr; we have found (manuscript in preparation) that as little as 6 nmol of EPO-generated HOBr can cause virtually complete lysis of a 2-cm^2 HUVEC monolayer, so that the amount of HOBr produced by 5×10^6 TNF-stimulated EOs is probably capable of causing significant cytolysis.

In previous studies of PMNs, TNF has been found to upregulate cell surface CR3 ($CD11_b$/CD18:$C3_{bi}$ receptor/adherence glycoprotein) expression, stimulate (>50-fold) phagocytosis of unopsonized zymosan particles by a CR3-dependent mechanism (2), and increase PMN adherence to HUVEC (1). To determine whether TNF also increases EO CR3 expression, we used FITC-conjugated Mol, a mAb with specificity for the $CD11_b$ α subunit of CR3,to perform flow cytometric analysis of purified (>95%) populations of PMNs and EOs isolated from the same donors ($n = 3$). As shown in Fig. 4 (*left*), EOs express Mol-reactive epitope to the same extent as PMNs before exposure to any known agonist of phagocyte activation, confirming previous observations by others (41). Moreover, EOs and PMNs are equally capable of upregulating $CD11_b$ expression, because in response to known agonists (1 μM FMLP for PMNs, 20 ng PMA for EOs), surface expression of the Mol-reactive epitope doubles (Fig. 4, *right*). However, on exposure to 100 U/ml TNF, responses of EOs and PMNs strikingly diverged: PMN $CD11_b$ expression doubles while that of EOs is totally unaffected (Fig. 4, *middle*). This surface receptor analysis was further corroborated in a functional assay, as TNF was found to be without effect upon EO uptake of unopsonized zymosan particles (not shown). TNF also failed to affect EO expression of LFA-1 and p150,95, two other members of the integrin family (not shown).

TABLE II

TNF Stimulation of EO O_2^- and HOBr Generation

Oxidant generated	EOs	EOs + TNF (100 U/ml)	EOs + HITNF (100 U/ml)	EOs + PMA (100 ng/ml)
nmol $O_2^-/10^6$ cells/18 h	4.4 ± 1.0	9.4 ± 3.2*	4.5 ± 2.8	22.5 ± 6.2*
nmol HOBr/10^6 cells/18 h	1.3 ± 1.0	2.9 ± 1.4*	1.6 ± 1.2	21.2 ± 4.0*

10^6 EOs were allowed to adhere to the bottom of 2-cm^2 FCS-coated plastic tissue culture wells in a final volume of 1 ml EBSS. Wells were supplemented with 75 μM cytochrome c ± 20 μg/ml superoxide dismutase for measurement of superoxide anion, and 32 μM sodium fluoresceinate and 1 mM NaBr for measurement of HOBr. After a 16-h incubation at 37° C, the tissue culture plates were spun 1,500 g for 5 min, and supernatant buffer was aspirated for spectrophotometric determination of O_2^- and HOBr, as described in Materials and Methods. HITNF, heat-inactivated TNF. Data presented ± SD.

* p vs. EO < 0.05.

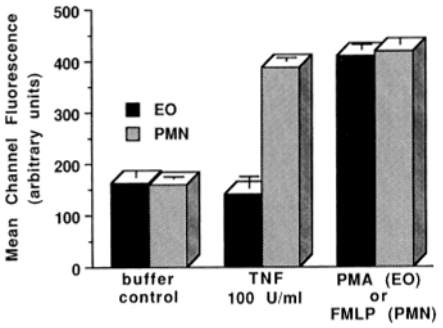

FIGURE 4. Flow cytometry analysis of PMN and EO CR3 expression detected by Mol (anti-$CD11_b$) mAb. 200,000 cell aliquots of EOs or PMNs, both purified to >95% homogeneity (n = 3), were incubated 15 min at 37°C before adding the indicated agonists, incubated another 30 min at 37°C., then exposed to FITC-Mol for 30 min at 4°C before flow cytometric analysis of 10,000 cells. Unlabeled EOs and PMNs had a mean channel fluorescence of <5. Identical gain settings were used to analyze PMNs and EOs. Agonists were TNF, 100 U/ml, PMA 20 ng/ml, and FMLP, 10^{-6} M. Error bars ± SEM.

Thus, in contrast to PMN, TNF stimulation of increased EO adhesion to HUVEC (15) occurs independently of increased surface CR3 expression. In PMNs, upregulated surface expression of these molecules reflects mobilization of intracellular pools (42), suggesting that TNF fails to induce EO degranulation (see Discussion) or that EO granules do not store integrins.

As well as stimulating various aspects of phagocyte function, TNF also directly affects diverse aspects of endothelial cell function, for example, their adhesiveness for PMNs (1), expression of HLA antigens (43), and production of granulocyte/macrophage-CSF (44). Because TNF induces oxidant damage and reduces antioxidant defenses in susceptible tumor cells (45), we determined whether TNF similarly diminishes antioxidant defenses in HUVEC. As shown in Table III, HUVEC monolayers exposed 18 h to 100 U/ml TNF release only slightly more ^{51}Cr than untreated monolayers when subsequently incubated 16 h with buffer alone, 10 ng/ml of PMA, or unstimulated EOs. In contrast, TNF (but not heat-inactivated TNF)-pretreated HUVEC monolayers release more than twice as much ^{51}Cr when subsequently exposed to EOs whose respiratory burst was stimulated by 10 ng/ml PMA. Because H_2O_2 has been shown to be the major oxidant species responsible for damage to HUVEC by PMA-stimulated PMNs (46), we compared the vulnerability of control and TNF-pretreated HUVEC to a range of reagent H_2O_2 concentrations. As shown in Fig. 5, TNF-pretreated HUVEC are modestly but significantly more susceptible to lysis by 75 μM and, especially, 133 μM H_2O_2. In three experiments, the concentration of H_2O_2 causing half-maximal ^{51}Cr release from TNF-pretreated endothelium was ~50% of that for control endothelium. Thus, exposure of HUVEC to subcytolytic and physiologically relevant concentrations of TNF significantly enhances its susceptibility to destruction by activated EOs and H_2O_2.

Discussion

Peripheral blood EOs, normally present at <400 cells/μl, may be greatly increased in such disorders as parasitic infestations, allergic or hypersensitivity states, vasculitis, and neoplasia (8). Unfortunately, chronic peripheral blood eosinophilia, irrespective of its cause, can be complicated by a morbid and often lethal clinical syndrome characterized by endocarditis, intracardiac capillary damage, and fleeting vasculitic skin rashes and pulmonic infiltrates, all of which reflect widespread endothelial cell damage (9, 10, 47). Immunohistochemical studies demonstrate (48) dense endothelial deposition of cytotoxic EO granule constituents, MBP, and EO cationic protein (ECP)

TABLE III

Vulnerability of TNF-exposed HUVEC to Damage by Activated EOs

Endothelial cell preparation	Specific ^{51}Cr release			
	EBSS buffer	PMA (10 ng/ml)	EOs (5×10^6)	EOs + PMA
Control HUVEC	0.0 ± 2.1	0.8 ± 0.3	5.3 ± 2.1	20.6 ± 5.4
HUVEC exposed 18 h to TNF (100 U/ml)	4.1 ± 2.1*	1.8 ± 0.4	7.3 ± 1.2	55.5 ± 2.2‡
HUVEC exposed 18 h to heat-inactivated TNF (100 U/ml)	3.9 ± 0.9*	ND	ND	20.2 ± 2.6

Monolayers of HUVEC were incubated 18 h in tissue culture medium alone (control), or in tissue culture medium supplemented with 100 U/ml TNF or 100 U/ml heat-inactivated TNF. Monolayers were then washed, labeled with ^{51}Cr, and incubated 16 h in 1 ml final volume EBSS containing the indicated additives before determining specific ^{51}Cr release. Data presented ± SD.

* $p < 0.05$ vs. control HUVEC + EBSS buffer.

‡ $p < 0.001$ vs. control HUVEC + PMA + EOs.

from the earliest stages of this disease onward, implicating a deleterious interaction between EOs and endothelium in the pathogenesis of this disease.

Our findings provide insights potentially relevant to such EO-mediated tissue damage, in that we have shown that TNF promotes EO toxicity towards human endothelium, activates the EO respiratory burst, and renders endothelium more vulnerable to oxidant damage. These findings raise the possibility that in the setting of chronic peripheral blood hypereosinophilia, increased serum levels of TNF might promote diffuse endothelial damage, including endocarditis. There are a number of clinical circumstances in which hypereosinophilia and elevated levels of TNF coexist. Perhaps the most common would be found in equatorial Africa or Southeast Asia, where filariasis, and consequently eosinophilia, is endemic together with Loeffler's endocarditis and endomyocardial fibrosis (9). Though it is not known whether filariasis per se is associated with elevated plasma levels of TNF, such common coendemic diseases as malaria and trypanosomiasis consistently cause elevated serum TNF levels (17). Elevated TNF and hypereosinophilia may also coexist in neoplasia, either naturally (17) or iatrogenically, as the result of IL-2 infusions, which cause increased lymphocyte secretion of TNF as well as high grade eosinophilia (49), or TNF infusions, which cause a modest eosinophilia (50). Of more widespread relevance, Veith and Butterworth (51) have demonstrated that monocytes derived from donors with moderate eosinophilia spontaneously secrete into supernatant culture

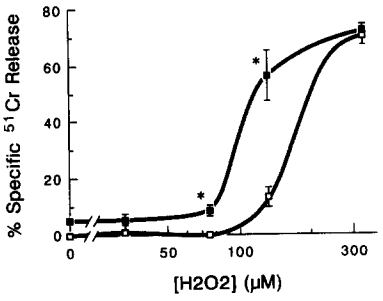

FIGURE 5. Vulnerability of TNF-treated HUVEC to damage by H_2O_2. HUVEC monolayers exposed 18 h to 100 U/ml TNF, then thoroughly washed, ^{51}Cr-labeled, and incubated another 16 h in 1 ml EBSS with the indicated concentrations of reagent H_2O_2. (□) Control HUVEC; (■) TNF-exposed HUVEC. An asterisk indicates cytotoxicity significantly different ($p < 0.05$) from that of control HUVEC. Error bars ± SD.

medium a factor capable of stimulating antibody-dependent cytotoxicity for the schistosomula of *Schistosoma mansoni*. A major component of this supernatant activity was subsequently found to be TNF (16). If these in vitro data can be extrapolated in vivo, peripheral blood eosinophilia may typically be accompanied by increased serum levels of TNF.

To validate our in vitro studies, we have considered three possible artifacts. First, TNF stimulation of EO damage to HUVEC could reflect endotoxin contamination of our rhTNF preparation. Although endotoxin alone in concentrations >1 μg/ml provokes minimal PMN damage to endothelial cells, pretreatment of PMNs with endotoxin in much lower doses (1–10 ng/ml) greatly potentiates ("primes") endothelial damage caused by subsequent addition of agonists such as 10^{-7} M FMLP (18). We consider it highly unlikely that endotoxin in our TNF preparation was responsible for its stimulation of EO toxicity to human endothelial cells because: (*a*) our TNF preparation contains <10 fg endotoxin/1,000 U and a several order of magnitude of excess polymyxin B (Table I) was without effect; (*b*) our TNF preparation, unlike endotoxin, loses its activity when heated for 15 min at 90°C (Tables II and III); and (*c*) anti-TNF rabbit serum attenuates TNF-stimulated EO toxicity for human endothelial cells, whereas control rabbit serum does not. We cannot, however, rule out the possibility that endotoxin contaminating our medium and buffers could somehow have "primed" EOs to respond to TNF.

Second, TNF-stimulated EO toxicity towards endothelium may be an artifact of prior exposure of EOs to FMLP during the isolation procedure. Yazdanbakhsh et al. (52) have shown that EOs prepared by a discontinuous Percoll gradient technique in the absence of FMLP activate their respiratory burst in response to FMLP, though to a much lower level than PMNs. In addition, pre-exposure of EOs to FMLP potentiated their subsequent response to PMA (52), suggesting that prior exposure to FMLP might likewise prime EOs to respond to other agonists, such as TNF. However, when we isolated EOs from peripheral blood by the alternative procedure described by Yazdanbakhsh et al., (52), they responded to 100 U/ml TNF with a similar degree of enhanced cytotoxicity towards endothelium as EOs isolated by our FMLP technique (250 ± 30% vs. 243 ± 20%; $n = 3$). Similarly, EOs isolated from a donor with primary hypereosinophilia, whose chronic 90–98% eosinophilia allowed purification on a one-step Percoll gradient ($d = 1.077$) without prior exposure to FMLP, showed a similar degree of enhanced cytotoxicity towards human endothelium in the presence of TNF (210 ± 63%; $n = 4$). Nonetheless, we acknowledge that since the majority of our EO donors had significant eosinophilia, we cannot be certain that our findings could be replicated using EOs from persons with normal EO counts. However, no correlation was evident between the magnitude of TNF stimulation of EO endothelial killing or oxidant production and degree of eosinophilia over a wide range from 4 to 39% (not shown).

Finally, it is possible that small numbers of PMNs contaminating our EO preparations were responsible for generating the increase in superoxide anion and HOBr measurable after exposure of EOs to TNF (Table II). We consider this highly improbable because: (*a*) PMN contamination of EO specimens varied between <1 and 15%, yet the degree of stimulation was similar in all the experiments (not shown); (*b*) the production of superoxide anion in these minimally contaminated EO preparations is (on a per cell basis) twice that reported by Larrick et al. (3) for purified

populations of PMN; (*c*) activated EOs, but not PMNs, produce HOBr instead of HOCl in the presence of 150 mM Cl^- and 100 μM Br^- (36); and (*d*) TNF stimulates NBT dye reduction in individual EOs (not shown).

In considering mechanisms by which TNF-exposed EOs provoke endothelial cell injury, we have shown that TNF stimulates surface-adherent EOs to produce superoxide anion and the EO-specific, cytotoxic oxidant HOBr; however our data do no unequivocally establish a role for these oxidants in causing endothelial cell damage. In fact, superoxide dismutase and catalase, which should theoretically "scavenge" superoxide anion and H_2O_2, had no effect upon TNF/EO-mediated endothelial cell killing (Fig. 2). However, this result does not rule out an important contribution by oxidants to the killing mechanism, since these high molecular weight scavenging proteins may not gain access to the space between endothelial cells and attached EOs. Moreover, any HOBr generated by EOs intracellularly could subsequently freely diffuse to cause target cell damage impervious to the scavenging effect of catalase. Indeed, Vissers et al. (53) have shown that PMNs adherent to glomerular basement membranes containing IgG aggregates produce considerable amounts of H_2O_2 in the contact area between the cells and target basement membrane but release no detectable extracellular (and therefore scavengeable) O_2^-. Moreover, Pincus et al. (54) have shown that supernatant medium from cultured blood mononuclear cells, which contains TNF (16), enhances EO ADCC towards schistosomula by a mechanism that is oxygen dependent yet not inhibitable by superoxide dismutase and catalase. Considering that PMN (40) and EO adherence to biologic surfaces potentiates TNF activation of the respiratory burst, "polarized" or contact-localized oxidant generation in a sequestered space, such as that described above, would serve a useful purpose to exclude potential scavenging substances and focus oxidant damage to an attached target while sparing surrounding host cells. Compatible with this hypothesis is the ability of TNF to stimulate EO toxicity towards endothelium in the presence of serum, which is normally a potent scavenger of reactive oxidants (EOs in EBSS, 6.6% specific ^{51}Cr release; EOs + 100 U/ml TNF in EBSS, 47.9%; EOs in EBSS + 10% FCS, 2.0%; EOs + TNF in EBSS + 10% FCS, 24.6%; TNF in EBSS + 10% FCS, 0.6%).

In addition to their potent oxygen-dependent cytotoxic capacities, EOs also use highly effective oxygen-independent killing mechanisms. For instance, EOs kill schistosomula by a peroxidase-dependent mechanism in the presence of oxygen (55), yet can kill just as effectively in an anaerobic environment (56). Oxygen-independent toxicity is mediated by EO-specific granule cationic proteins, including MBP, ECP, and eosinophil-derived neurotoxin, which are all directly cytolytic for a variety of metazoan and mammalian cells (57). If TNF could induce degranulation of EOs, then these cationic proteins might damage adjacent endothelial cells independent of oxidant mechanisms. However, we find that only nonphysiologic, high ($\geq$10,000 U/ml) concentrations of TNF induce EO degranulation, assayed as release of EPO into supernatant buffer (not shown). Nonetheless, the possibility remains that at the lower TNF doses used in the course of our studies, localized degranulation, similar to localized oxidant production as described above, contributes to EO endothelial cell damage. The capacity of heparin, which blocks the toxicity of MBP (28), to inhibit TNF/EO-mediated endothelial cytotoxicity (Fig. 2) suggests this may be so.

In addition to stimulating EO oxidant production, we also find TNF directly affects endothelial cells, enhancing their vulnerability to damage by PMA-stimulated EOs, perhaps, in part, by increasing their susceptibility to lysis by H_2O_2. Alternatively, enhanced cytolysis of TNF-exposed endothelium may reflect increased adhesion of EOs, since TNF treatment of endothelium is known to enhance subsequent EO attachment by a CD11/CD18 leukocyte adherence glycoprotein-dependent mechanism inhibitable by mAb 60.3 (15). However, we doubt this mechanism is important, because in studies not shown, this antibody (50 μg/ml) does not attenuate the increased cytolysis of TNF-treated endothelium by PMA-stimulated EOs. Instead, we propose that TNF treatment of human endothelium decreases its antioxidant defenses, thereby rendering it more susceptible to killing by EOs activated by PMA, a potent respiratory burst stimulant. That TNF-exposed HUVEC are also more damaged by H_2O_2 (Fig. 5) and FMLP- and PMA-stimulated PMN (not shown) supports this interpretation. Moreover, Varani et al. (58) have previously shown that rat pulmonary artery endothelium pre-exposed to TNF is more vulnerable to damage by C5a- and PMA-activated PMN, raising the possibility that this phenomenon may be a generalized response of endothelium to TNF. Also compatible with our hypothesis are the data of Zimmerman et al. (45), which show that in the L929 tumor line, acute exposure to TNF causes accumulation of oxidized glutathione (GSSG) and a drop in the glutathione/GSSG ratio, indicating TNF mediates both ongoing oxidant production and diminished oxidant buffering capacity. As it is unlikely that the modestly enhanced vulnerability of TNF pretreated endothelium to bolus H_2O_2 (Fig. 5) accounts entirely for its increased susceptibility to damage by phagocytes whose respiratory burst has been activated (Table III), we are currently characterizing the effect of TNF exposure upon several endothelial cell antioxidant defenses, including NADPH, catalase, superoxide dismutase, GSH, and glutathione peroxidase levels.

Regardless of the precise mechanism involved, our observation that exposure of human endothelial cells to TNF enhances their vulnerability to damage by activated EOs, PMNs, and H_2O_2 has obvious implications for the pathogenesis of clinical syndromes involving phagocyte-dependent endothelial damage. For instance, in chronic hypereosinophilic states associated with widespread endothelial damage, peripheral blood EOs are activated, spontaneously producing cytotoxic oxidants (59). In this setting, elevated serum TNF could both further activate EO oxidant production and concomitantly render endothelium more vulnerable to damage by these same cells, thereby producing a synergistic effect upon endothelial cell toxicity. Endotoxin-induced shock and the localized Shwartzmann reaction, both mediated at least in part by TNF (5, 60), may similarly reflect the capacity of TNF not only to activate phagocytes but also to diminish the capacity of endothelial cells to withstand their onslaught.

Finally, we note that TNF stimulation of EO toxicity towards unopsonized human endothelium occurs at TNF concentrations orders of magnitude more (100 vs. 0.01 U/ml) than those that stimulate ADCC towards antibody-opsonized schistosomula (16). This difference may indicate the existence of a wide therapeutic "window" of concentrations in which TNF normally functions beneficially to aid EOs rid the host of parasites. However, abnormally elevated TNF levels, such as occur (5) in response to chronic intracellular parasitic infestations, malignancy, or endotoxinemia, may provoke circulating EOs to damage host endothelium with adverse clinical consequences.

Summary

Eosinophils (EOs) participate in a variety of inflammatory states characterized by endothelial cell damage, such as vasculitis, pneumonitis, and endocarditis. We find that 100 U/ml TNF-α/cachectin (TNF), a concentration attainable in the blood of humans with parasitic infestations, stimulates highly purified populations of EOs to damage human umbilical vein endothelial cells (HUVEC), a model of human endothelium. This TNF-dependent EO cytotoxicity is strongly inhibited by heparin and methyprednisolone but unaffected by the platelet-activating factor antagonist BN52012 or scavengers of superoxide anion and H_2O_2, superoxide dismutase and catalase. However, addition of a physiologically relevant concentration of Br^- (100 μM) enhances EO/TNF damage to HUVEC, implicating the possible participation of EO peroxidase (EPO) in the killing mechanism. EOs adherent to FCS-coated plastic wells more than double their production of superoxide anion and the cytotoxic EPO-derived oxidant HOBr when exposed to TNF, showing that TNF activates the respiratory burst of EOs attached to a "physiologic" surface. Unlike PMNs, EOs were not irreversibly activated to kill unopsonized endothelium by previous exposure to TNF, and did not degranulate or upregulate CR3 expression as detected by Mol in the presence of 100 U/ml TNF. HUVEC exposed 18 h to TNF were considerably more susceptible to lysis by PMA-activated EOs and reagent H_2O_2, demonstrating a direct effect of TNF upon endothelium, perhaps through inhibition of antioxidant defenses. These findings suggest that abnormally elevated serum levels of TNF may provoke EOs to damage endothelial cells and thereby play a role in the pathogenesis of tissue damage in hypereosinophilic states.

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