TUMOR NECROSIS FACTOR/CACHECTIN INCREASES PERMEABILITY OF ENDOTHELIAL CELL MONOLAYERS BY A MECHANISM INVOLVING REGULATORY G PROTEINS

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Endothelium serves as the cellular barrier retaining blood components within the intravascular space (1). This function is compromised in many pathophysiologic states. Most notably, the profound alterations in endothelial cell barrier function specifically induced by host mediators released in response to bacterial LPS contribute importantly to the pathogenesis of endotoxic/septic shock.

The cytokine tumor necrosis factor/cachectin (TNF), one of several host mediators synthesized by macrophages after exposure to LPS, has been shown to be a central mediator of the shock state in Gram-negative sepsis (2, 3). Infusion of TNF into animals produces many of abnormalities characteristic of *Escherichia coli*/LPS toxemia, including vascular leakage due to loss of barrier function (2, 4). Vascular endothelium has been shown to be a direct target for the action of TNF, which causes modulation of many endothelial cell functions, such as coagulant activity and immunologic properties (2). Recent studies (5–7) suggest that the normal barrier function of the endothelium can also be altered directly by TNF. These considerations have led us to examine the effect of TNF on endothelial permeability. The results indicate that within 1–3 h after exposure to TNF, permeability of cultured endothelial monolayers to macromolecular and lower molecular weight solutes increased markedly; this effect was temporally correlated with alterations in the cytoskeleton and cell shape, and the formation of intercellular gaps. Pertussis toxin blocked both the change in cell shape/cytoskelton and the increase in permeability, indicating that a pertussis toxin-sensitive G protein critically regulates this TNF-induced response. In contrast, other TNF-induced changes in endothelial cell function, such as perturbation of certain cell surface coagulant properties, were not affected by pertussis toxin. These data suggest the presence of more than one TNF-dependent signal transduction pathway within endothelial cells: a pathway involving a pertussis toxin-sensitive G protein leads to increased vascular permeability, whereas, a separate mechanism underlies the modulation of other cellular properties by TNF.

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Materials and Methods

Reagents. Recombinant human TNF ($\sim 10^8$ U/mg) was generously provided by Dr. P. Lomedico of Hoffmann-La Roche, Inc. (Nutley, NJ). This preparation was homogeneous on SDS-PAGE and distinct from lymphotoxin. Bovine protein S, protein C, thrombin, and antithrombin III were purified by previously described methods (8–11) and protein S was radioiodinated by the lactoperoxidase method (8,000 cpm/ng) (12). Fatty acid–free bovine albumin (Sigma Chemical Co., St. Louis, MO) was also radioiodinated by the lactoperoxidase method (5,000 cpm/ng). After radiolabeling, tracers were de-salted by gel filtration and subjected to extensive dialysis. Both tracers were >95% precipitable in TCA (15%). Tritiated inulin (271 mCi/g) and sorbitol (24 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Monospecific rabbit antiserum to human TNF, generously provided by Dr. D. Blohm (Knoll, FRG), was used to prepare IgG fraction and coupled to affi-gel 10 (Bio-Rad Laboratories, Richmond, CA) as described previously (13). Antibody to bovine tissue factor and purified bovine tissue factor were generously provided by Dr. R. Bach (Mt. Sinai School of Medicine, New York, NY) (14). Mouse mAb to plakoglobin was obtained from ProGen (Heidelberg, FRG). Lactate dehydrogenase activity was measured using a kit purchased from Sigma Chemical Co.

Cell Culture. Bovine aortic endothelial cells were isolated from calf aortas as described (15) and were grown in MEM (Gibco Laboratories, Grand Island, NY), supplemented with penicillin-streptomycin (50 U/ml; 50 μg/ml), glutamine (1%), and FCS (15%) (HyClone Laboratories, Logan, UT). Cells were grown in a humidified, 5% CO_2 atmosphere and were transferred b trypsinization. For experiments, cells (passages 2–6) from different aortas were grown to confluence ($\sim 10^5$ cells/cm^2) in 35-mm dishes, 24-well, 96-well, or Transwell plates (6.5-mm polycarbonate membranes, with a pore size of 0.4 μm, mounted on polystyrene inserts; Costar, Cambridge, MA). For morphologic studies, cells were grown on coverslips inserted in 35-mm dishes. Cultures were characterized as endothelial by the formation of cobblestone-like monolayers, contact inhibition at confluence, and the presence of Factor VIII–related antigen, protein S, and thrombomodulin (15–18). In some studies, endothelial cultures were preincubated with pertussis toxin (List Biologicals, Campbell, CA) for 18 h before the start of an experimental protocol. The range of pertussis toxin concentrations used (0.1–10 ng/ml) was based upon earlier studies indicating that this dose range is associated with progressive ADP ribosylation and inactivation of the endogenous pertussis toxin–sensitive G protein in endothelial cultures (19). At a concentration of 10 ng/ml of pertussis toxin, >98% of the endogenous pertussis toxin–sensitive G protein in the culture is ADP ribosylated. Moreover, higher concentrations of pertussis toxin (100 ng/ml) appeared to have a direct toxic effect on the cultures, perhaps due to the known action of high concentrations of the toxin to directly activate cells (20), and were not used.

Permeability Studies. Confluent monolayers (5–6 d after plating) in Transwell inserts were washed with Earle's balanced salt solution (0.5 ml), and either MEM containing fatty acid–free BSA (1%; Sigma Chemical Co.) or medium containing FCS (5%) was added to each compartment (to avoid an oncotic gradient). Final volumes present in the inner and outer (corresponding to upper and lower, respectively) chambers were adjusted to yield no hydrostatic gradient across the monolayer. Radiolabeled markers (albumin, inulin, or sorbitol) were then added in trace concentrations (150 ng/ml, 3 μg/ml, and 38 ng/ml, respectively) to the upper chamber. TNF or heat-treated TNF (and other agents such as cycloheximide or actinomycin D) was added simultaneously with the tracers unless indicated otherwise. For assays, the chambers were incubated at 37°C in a 5% CO_2 atmosphere with continuous agitation, and samples (0.005 ml) were withdrawn at the indicated times. The volume in the chambers was not changed significantly (<5%) as a result of sampling of wells during the experiment. Transport of tracers from the inner to outer chamber, i.e., across the endothelial monolayer, was determined by dividing radioactivity in the outer well by radioactivity in the inner well (measured from duplicate 0.005-ml samples from each well). Wells were also sampled within 5 min of tracer addition to establish a t_o background level, and monolayers displaying evidence of leakage were discarded. Since experiments with ^{125}I-albumin were carried out in the presence of a large excess of unlabeled ligand, the observed passage of tracer across the monolayer was not due to a receptor-mediated process.

Immunocytology. Cell monolayers grown on coverslips were fixed briefly (1–2 min) in PBS, pH 7.2, containing 3.5% formalin and 0.1% NP-40, then in 3.5% paraformaldehyde without NP-40 for an additional 5 min and washed in PBS. For visualization of F-actin, coverslips were incubated with rhodamine-conjugated phalloidin (Molecular Probes, Junction City, OR) for 30–45 min, washed in PBS, and mounted in Gelvatol containing 1 mg/ml *p*-phenylenediamine. Mounted coverslips were examined in a microscope (Dialux 20; E. Lietz, Inc., Wetzlar, FRG) and recorded on Kodak Tri-X film. The protocol for visualization of plakoglobin was similar to that described previously for protein S (17).

EM. Monolayers grown on glass coverslips were fixed for scanning EM in cacodylate buffer (0.1 M, pH 7.2) containing glutaraldehyde (2.5%) and sucrose (2%) for 45 min, and then washed three times in buffer followed by post-fixation in buffer containing osmium tetroxide (2%). Fixed monolayers were dehydrated in ethanol, transferred to Pel-Dry (Ted Pella Inc., Redding, CA), and dried by sublimation in a fume hood. Dried monolayers were coated with gold palladium and viewed in a scanning electron microscope (T-300, Joel, Peabody, MA).

Assays of Endothelial Cell Coagulant Properties. The study of protein S released by endothelium was carried out by a previously described method (17, 19). In brief, confluent endothelial monolayers (9.6 cm^2) were washed four times over a period of 1 h at 37°C. This resulted in dissociation of cell surface protein S before the start of an experiment designed to detect release of intracellular protein S from endothelium in response to TNF. After washing, 1 ml of MEM containing HEPES (10 nM; pH 7.4) and BSA (5 mg/ml) were added in the presence or absence of mediators. Aliquots of culture supernatant were obtained at the indicated times and assayed for protein S antigen as described previously (19). When low levels of protein S antigen were detected in samples, assays were repeated after 10-fold concentration.

Induction of endothelial cell tissue factor activity was studied by incubating endothelium with TNF (0.5 nM) in MEM containing 1% FCS. Tissue factor activity of endothelial cells was then measured using a coagulant assay after 8 h, as described previously (21). The specificity of this assay for measurement of endothelial cell procoagulant activity induced by TNF was confirmed by studies using rabbit antibovine tissue factor IgG (14). Preincubation of cells with this IgG (10 μg/ml) for 1 h at 37°C before carrying out the tissue factor assay blocked the procoagulant activity observed in TNF-stimulated endothelium. The amount of tissue factor activity present in samples was determined by comparison of the clotting time with a standard curve constructed using the same tissue factor assay and purified bovine tissue factor (14).

TNF-induced suppression of endothelial cell thrombomodulin was studied by incubating confluent monolayers (0.32 cm^2) with TNF (0.5 nM) for 8 h at 37°C. Thrombomodulin activity was then assessed by measuring thrombin-mediated protein C activation over the endothelial cell monolayers as previously described (22). In brief, samples were incubated with thrombin (0.2 U/ml) and protein C (90 μg/ml) for 30 min at 37°C. Antithrombin III (0.2 mg/ml) and heparin (1 U/ml) were added next, and then hydrolysis of the chromogenic substrate Lys-Pro-Arg-*p*-nitroanilide (Spectrozyme, American Diagnostica, NY, NY) was monitored at 405 nm. The amount of activated protein C formed was determined by comparison with a standard curve made with known amounts of activated protein C.

Results

Cell Shape and Cytoskeleton. Normal endothelium forms a contiguous monolayer of closely apposed polygonal cells, each of which has a prominent circumferential, peripheral band of actin microfilaments and a more variable display of thin, randomly oriented stress fibers (Fig. 1 *A*). Cells are closely apposed about their entire circumference, being secured in place by junctional complexes of the plasmalemma, chiefly, actin microfilament-associated maculae and zonulae adherents, characterized by their content of plakoglogin (23) (Fig. 1 *F*). Within 30–60 min of exposure to TNF (5 nM) (the latter concentration of TNF was used in this study since, as described below, it results in maximal TNF-induced modulation of endothelial per-

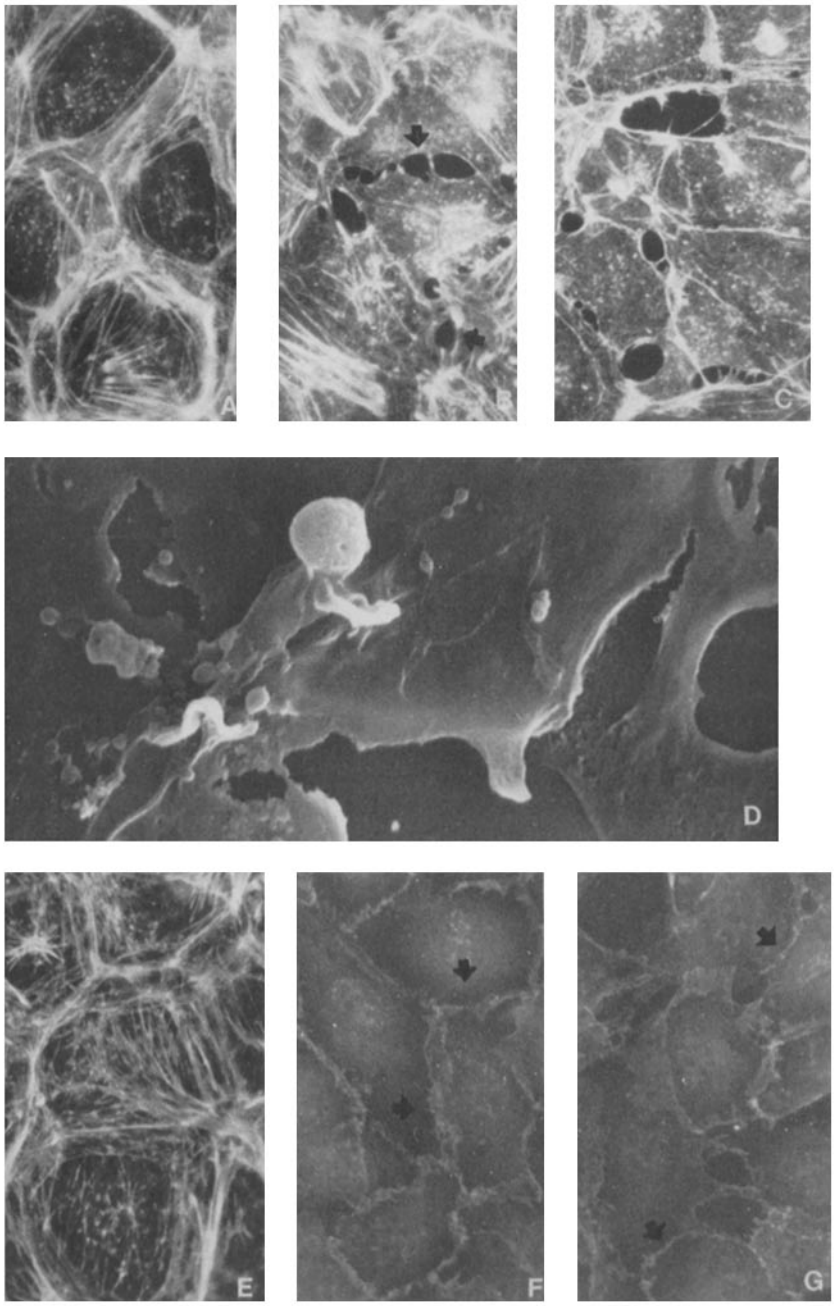


FIGURE 1. TNF-induced perturbation of cell shape/cytoskeleton. Monolayers of cultured endothelial cells were incubated in the presence or absence of TNF for the indicated times. The actin-based cytoskeleton was then visualized by rhodamine phalloidin staining (*A–C, E*) and plakoglobin distribution was displayed with a mouse mAb visualized with FITC-conjugated goat anti-mouse antibody (*F–G*). Cell shape was also assessed by scanning EM (*D*). (*A*) Control monolayer; (*B*) cultures exposed to TNF (5 nM) for 90 min. Arrows represent areas of lateral cell margin retraction; (*C*) cultures exposed to TNF (5 nM) for 24 h; (*D*) scanning electron micro-

meability), peripheral actin bands become indistinct and disappear, while at the same time, the central stress fibers in most of the cells become transiently more numerous and prominent, and subsequently condense, shorten, and appear to fuse. By 60–90 min of treatment with TNF, these stress fibers have contracted into dense microfilamentous (actin) masses. Cytoskeletal changes occur concomitant with the onset of mutual cell retraction, which results in the formation of intercellular gaps exposing the underlying matrix (Fig. 1 *B*). These processes continue slowly, and at later times (4–24 h), gaps between the retracted cells are larger and more numerous (Fig. 1 *C*). The retracted cells remain apposed and adherent over wide sectors, in which the arrangement of adhaerens junctions, identified in Fig. 1 *G* by their plakoglobin, appear relatively intact. However, the immunocytochemically detected plakoglobin of many sectors of membrane bordering the gaps becomes attenuated and may disappear (Fig. 1 *G*). All cellular margins are sharply outlined by actin of the plasma membrane cytoskeleton, which appears to become augmented at later times of exposure to TNF (Fig. 1 *C*). The intercellular gaps are well shown in the scanning electron microscope, which also reveals many protrusions on the upper (abbasal) surfaces of TNF-treated cells (Fig. 1 *D*), membrane-bound excrescences that are stuffed with microfilamentous actin. Throughout these events, the microtubule array appears unaffected, save for apparently passive displacements.

TNF-induced perturbation of endothelial ultrastructural characteristics is not a consequence of nonspecific toxicity; there was neither significant release of LDH into the medium, nor permeability to trypan blue, nor detachment of cells from the growth surface. Furthermore, these perturbations of endothelial cell shape and cytoskeletal integrity are reversible (Fig. 1 *E*). This was established by exposing cultures to TNF for 4 h, washing, and transferring the cultures to fresh medium. At 36 h, the cells had re-established a contiguous monolayer with few evident gaps, and the cytoskeleton displayed prominent marginal bands with few central stress fibers (compare Fig. 1, *E* and *A*).

Barrier Function. The actions of TNF to induce cytoskeletal rearrangements and cell retraction could lead to changes in endothelial barrier function. To test this directly, bovine aortic endothelial cells were cultured on microporous (0.4-μm) polycarbonate membranes. Monolayers formed on these membranes are capable of restricting diffusional passage of molecular markers as compared with membranes alone or confluent monolayers of bovine fibroblasts (Fig. 2). Incubation of endothelium with TNF (100 ng/ml; ~5 nM) led to a steady increase in the passage of tracer molecules across the monolayers (Fig. 3, *A–C*). Tracer solutes of a range of molecular weights/sizes were used (^{125}I-albumin, $M_r \approx 67000$; [^{3}H]inulin, $M_r \approx 5000$; [^{3}H]sorbitol, $M_r \approx 500$) in order to better characterize the nature of the diffusional defect. The TNF-induced increase in permeability to sorbitol was detectable by 60 min, whereas the enhanced permeability to larger molecular weight solutes (albumin and inulin) was detectable

graph of cells exposed to TNF (5 nM) for 4 h; (*E*) cultures exposed to TNF (5 nM) for 4 h, rinsed, and then maintained in fresh medium for 36 h; (*F*) localization of plakoglobin at the lateral margins (*arrows*) of confluent control endothelial cell monolayers; (*G*) distribution of plakoglobin after TNF (5 nM) for 4 h. Large sectors of the lateral cell margins (*arrows*) maintain a relatively intact plakoglobin distribution. Details of methods are described in the text. *A–C* and *E–G*, × 600; *D*, × 6,500.

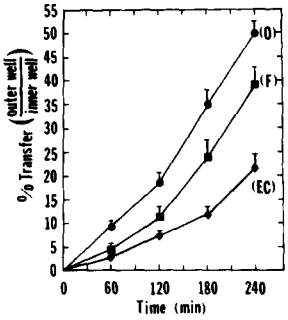


FIGURE 2. Barrier function of monolayers grown on polycarbonate membranes. Membranes alone (*O*) or with confluent monolayers of endothelial cells (*EC*) or bovine fibroblasts (*F*) were incubated with [^{3}H]sorbitol, and transfer of tracer across the membrane was determined at the indicated times as described in the text. Mean ± SD is shown.

at 120 min (Fig. 3 *A–C*). Permeability to all three tracers continued to increase steadily and progressively up to 4–8 h, ultimately attaining rates 1.5–2 times greater than the basal control rate. The changes in permeability induced by TNF were sustained for up to 24 h thereafter (data not shown).

The effect of TNF on monolayer permeability was concentration dependent. Monolayer permeability to all three tracers was half-maximal at a TNF concentration of ~0.5 nM and reached an apparent maximum by 5 nM (Fig. 3, *D–F*). This dose dependence roughly parallels the binding parameters previously reported for occupancy of TNF receptors on endothelium (13) and other cell types (24–29) in which the affinity of radioiodinated TNF for the cell surface was ~0.2–0.6 nM.

The effects of TNF on endothelial barrier function are specific and not due to the presence of any contaminant potentially present in the preparation (LPS, for example, has been shown to be directly toxic to bovine endothelial cells [30]). A TNF stock solution (20 nM) depleted of TNF by chromatography on an affigel 10

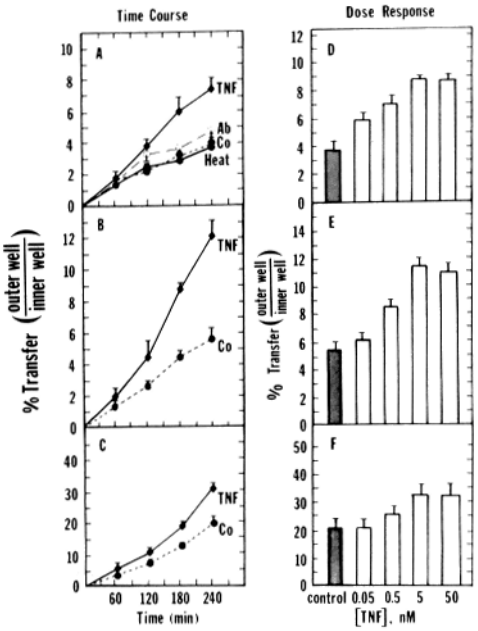


FIGURE 3. Effect of TNF on endothelial cell monolayer permeability to ^{125}I-albumin, [^{3}H]inulin, and [^{3}H]sorbitol. (*A–C*) Time Course, cultures were incubated either with ^{125}I-albumin (*A*), [^{3}H]inulin (*B*), or [^{3}H]sorbitol (*C*) for the indicated times in the presence (◆) or absence (●) of TNF (5 nM), and transfer of tracer across the monolayer was determined as described. Additional controls included in *A*: (*Ab*) the TNF preparation was pre-absorbed with a column of immobilized anti-TNF IgG; and (*Heat*) TNF was heat inactivated before use (see text for details). (*D–F*) Dose response: monolayers were incubated with ^{125}I-albumin (*D*), [^{3}H]inulin (*E*), or [^{3}H] sorbitol (*F*) in the presence of various TNF concentrations (0.05–10 nM) for 4 h and barrier function was determined. Control (*darkened bar*) denotes monolayers to which no TNF was added. The mean ± SD is shown.

column with immobilized IgG from a monospecific anti-human TNF antiserum (13) did not induce increased albumin permeability across monolayers (Fig. 3 *A*). In contrast, TNF solutions passed through a similar column with nonimmune IgG-altered permeability of the monolayer to albumin in a manner identical to native TNF. Furthermore, heat-treated TNF, which is biologically inactive (31), did not alter permeability. Taken together, these data indicate that TNF is the active species promoting diffusion, not contaminating endotoxin or other substances.

Since the half-life of TNF in the circulation is measured in minutes (32), we wondered whether short exposures to TNF lead to more sustained alterations in endothelial barrier function. To address this question, endothelium was exposed to TNF (1 nM) for 15 min at 37°C, extensively washed to remove unbound material, and permeability was assessed at 4 h. Monolayers treated in this fashion displayed increased permeability to ^{125}I-albumin and could not be distinguished from monolayers exposed to TNF continuously for 4 h (Fig. 4 *A*). Similar results were observed with inulin and sorbitol. These studies indicate that TNF binds to its endothelial cell receptors rapidly and initiates a series of events that lead to increased permeability of monolayers. Continued presence of cytokine in the supernatant is not required to maintain the response. Although only brief exposure to TNF induces the endothelial cell response, recovery of barrier function required considerable time (Fig. 4 *B*). After exposure to TNF for 4 h, an additional 36 h was required for permeability to reach pretreatment levels.

To examine whether the effect of TNF to enhance endothelial permeability required de novo protein synthesis, endothelial monolayers were exposed to TNF in the presence of inhibitors of protein and RNA synthesis (Table I). Neither cycloheximide (10 μg/ml) nor actinomycin D (1 μg/ml) impaired the actions of TNF to enhance monolayer permeability. Higher concentrations of these inhibitors were directly cytotoxic, precluding assessment of permeability changes. However, the concentrations of inhibitors tested did result in >90% decrease in total protein (based on [^{3}H]leucine incorporation) and RNA (based on [^{3}H]uridine incorporation) synthesis. These results suggest that the effect of TNF to increase albumin diffusion across endothelium is not dependent upon de novo protein synthesis and contrasts with other cellular effects of TNF that do require the biosynthetic apparatus.

Coagulant Properties. Changes in endothelial permeability can result in perturbation of the coagulation mechanism. On the one hand, plasma constituents, such

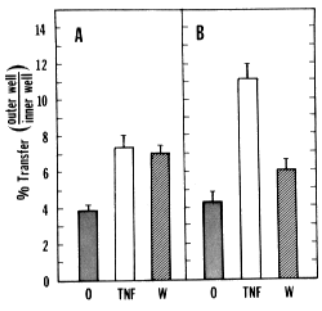


FIGURE 4. TNF-mediated perturbation of endothelial cell barrier function: the effect of brief exposure of cultures to TNF and recovery. (*A*) Effect of brief exposure of monolayers to TNF. Cultures were either incubated with TNF (5 nM) for 15 min, followed by 10 washes, and placed in fresh medium for 4 h (*W*) or medium containing TNF for 4 h (*TNF*). Control cultures were not exposed to TNF (*O*). ^{125}I-albumin passage across monolayers was determined by incubating tracer with cultures for 2 h. (*B*) Recovery. Cultures were exposed to TNF (5 nM) for 4 h (*TNF*) and then washed (four times over 30 min) and incubated with fresh medium for 36 h (*W*). Controls (*O*) were not exposed to TNF. Permeability was determined at the indicated times by assessing passage of ^{125}I-albumin across the monolayer during a 4-h incubation after the indicated pretreatment. Details of experimental procedure are described in the text. The mean ± SD is shown.

TABLE I

Effect of Cycloheximide and Actinomycin D on TNF-induced Modulation of Endothelial Cell Barrier Function

Condition	Percent ^{125}I-albumin transfer
Control (no addition)	4.1 ± 0.2
Cycloheximide alone	4.2 ± 0.2
Actinomycin D alone	4.7 ± 0.2
TNF	6.9 ± 0.6
TNF + cycloheximide	7.1 ± 0.7
TNF + actinomycin D	7.0 ± 0.6

Endothelial cell monolayers were incubated for 4 h in either normal medium (control), medium supplemented with cycloheximide (10 μg/ml) alone, actinomycin D (1.0 μg/ml) alone, TNF (5 nM) alone, TNF (1 nM) and cycloheximide (10 μg/ml), or TNF (1 nM) and actinomycin D (1 μg/ml). Passage of ^{125}I-albumin across monolayers was assessed by adding tracer to the chamber above the monolayers at the time TNF/drugs were added over the 4-h period. The mean ± SD is shown.

as clotting factors, are lost from the intravascular space. Specifically, protein S, an anticoagulant cofactor that functions effectively on the surface of quiescent endothelium (33), was observed to pass through TNF-treated monolayers (in a manner analogous to that observed in studies with radioiodinated albumin described above). Loss of protein S from the vascular lumen could potentially lead to attenuation of an intravascular antithrombotic mechanism. Since protein S is synthesized and stored in endothelium (17), one possible compensatory mechanism to counterbalance loss of plasma protein S could involve TNF-stimulated release of protein S (6). Incubation of TNF with monolayers did lead to release of protein S into the supernatant in a time- and dose-dependent manner. The effect of TNF on endothelial cell protein S release was fully expressed by 40 min (FIg. 5 *A*); a half-maximal effect occurred at a concentration of ~100 pM (Fig. 5 *B*). In a previous study we found that release of endothelial cell protein S in response to norepinephrine could be blocked by pertussis toxin. Those results led us to examine whether pertussis toxin had a

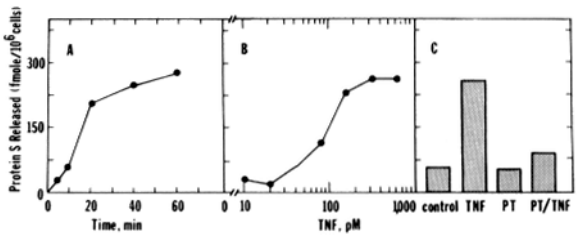
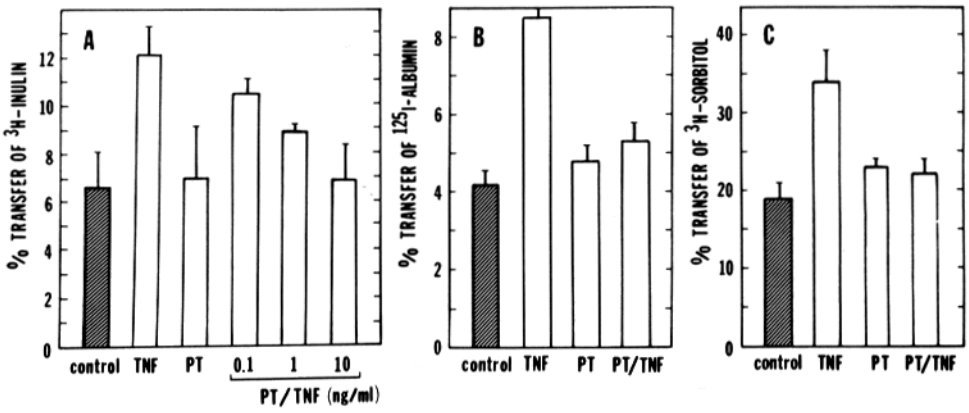


FIGURE 5. Release of protein S from endothelial cells: effect of TNF and pertussis toxin. (*A*) Time course. After removal of cell surface protein S, endothelial monolayers were incubated with TNF (1 nM) and aliquots of supernatant were removed at the indicated times and assayed for protein S antigen. (*B*) Dose response. Endothelium was incubated with varying concentrations of TNF (10–1,000 pM) for 50 min and samples of culture medium were assayed for protein S antigen. (*C*) Effect of pertussis toxin. Endothelial cell monolayers were preincubated in complete medium alone or in the presence of pertussis toxin (10 ng/ml) for 18 h at 37°C. Then cultures were exposed to TNF (200 pM) or fresh culture medium alone and supernatants were assayed for protein S antigen after 50 min. The mean of triplicates is shown and SD was <15% in each case. Details of experimental procedures are described in the text. *Control*, cultures exposed to culture medium alone; *TNF*, cultures preincubated in normal growth medium and then exposed to TNF; *PT*, cultures preincubated with pertussis toxin and then incubated in normal medium; *PT/TNF*, cultures preincubated with pertussis toxin and then exposed to TNF.

similar effect on TNF-induced release of protein S. Preincubation of cultures for 18 h with pertussis toxin (10 ng/ml), a protocol previously found to be associated with >98% ADP ribosylation and inactivation of the endogenous pertussis toxin-sensitive G protein in endothelial cultures (19), markedly attenuated TNF-induced release of protein S (Fig. 5 *C*).

Role of the Pertussis Toxin-sensitive G Protein. Studies demonstrating that pertussis toxin inhibits the action of TNF to release endothelial cell protein S (19) support a role for a pertussis toxin-sensitive G protein in signal transduction at the TNF receptor. We examined whether other effects of TNF on endothelial cell physiology are similarly dependent upon a pertussis toxin-sensitive intracellular transduction mechanism. Cultures were preincubated for 24 h with a range of pertussis toxin concentrations previously shown to induce partial (0.1-1 ng/ml) or complete (10 ng/ml) ADP ribosylation and inactivation of the endogenous pertussis toxin-sensitive G protein (19). These studies indicated that pertussis toxin inhibits the effect of TNF to augment passage of [^{3}H]inulin across endothelial monolayers in a dose-dependent fashion (Fig. 6 *A*). Moreover, the dose-response relationship for the effect of pertussis toxin to inhibit this TNF response and to ADP ribosylate the endogenous



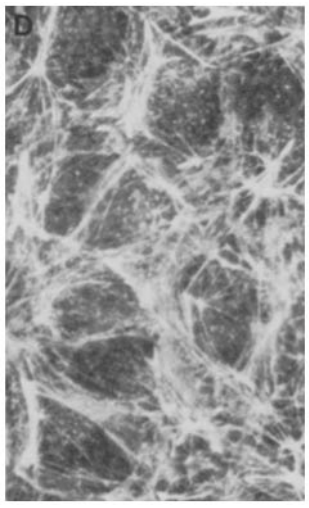


FIGURE 6. Effect of pertussis toxin on TNF-induced perturbation of endothelial cell barrier function and cytoskeleton/cell shape. Cultures were preincubated for 18 h with growth medium alone or medium supplemented with pertussis toxin at the indicated concentration (0.1–10.0 ng/ml), after which they were placed in medium alone (*control*), medium containing TNF (*TNF*), pertussis toxin (*PT*), or pertussis toxin and TNF (*PT/TNF*). Passage of tracers, as indicated ([^{3}H]inulin, *A*; ^{125}I-albumin, *B*; [^{3}H]sorbitol, *C*), through monolayers, was measured over 4 h starting after the 18-h preincubation period. (*B* and *C*) The concentrations of TNF and pertussis toxin were 5 nM and 10 ng/ml, respectively. The mean ± SD is shown. Details of experimental procedure are described in the text. *Control,* cultures exposed to normal growth medium alone; *TNF*, cultures preincubated in normal growth medium and then exposed to TNF for 4 h; *PT*, cultures preincubated with pertussis toxin and then incubated in normal culture medium; *PT/TNF*, cultures preincubated with pertussis toxin and then exposed to TNF (5 nM). (*D*) Cytoskeleton/cell shape. Rhodamine phalloidin staining of the actin-based cytoskeleton after exposure of cultures to pertussis toxin (10 ng/ml) and TNF (5 nM) as described above. This micrograph should be compared with Fig. 1, *A* and *B*. (× 600).

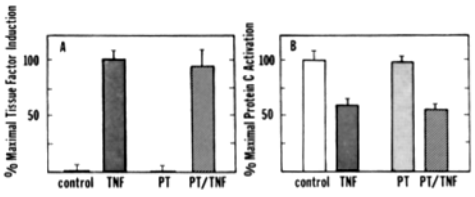


FIGURE 7. Effect of pertussis toxin on TNF-induced modulation of endothelial cell coagulant properties: induction of tissue factor (*A*) and suppression of thrombomodulin (*B*). Cultures were preincubated for 18 h with either complete medium or medium supplemented with pertussis toxin (10 ng/ml). Then cultures were placed in fresh medium with either TNF (0.5 nM) or no further addition was made. Assays of tissue factor and thrombomodulin activity were made after an additional 8 h of incubation. Data is shown as percent maximal tissue factor induction (maximal tissue factor induction is that observed with TNF alone and corresponds to 38 fmol/10^6 cells) and percent maximal protein C activation (maximal protein C activation is that observed on endothelium incubated with normal medium and corresponds to 25 μg/ml/30 min). The mean ± SD is shown and details of experimental procedure are described in the text. *control*, cultures exposed to normal growth medium alone; *TNF*, cultures preincubated in normal growth medium and then exposed to TNF; *PT*, cultures preincubated with pertussis toxin and then incubated in normal culture medium; *PT/TNF*, cultures preincubated with pertussis toxin and then incubated with TNF.

G protein correlated highly, providing strong evidence for a role for a pertussis toxin-sensitive G protein in this TNF response. Pertussis toxin also blocked the increase in endothelial permeability to radiolabeled albumin and sorbitol induced by TNF (Fig. 6, *B–C*). Consistent with these results, cytokine-mediated perturbations of the actin-based cytoskeleton and cell shape were also inhibited by pertussis toxin, albeit not completely (Fig. 6 *D*). These results constitute strong evidence that these acute changes in endothelial cell physiology induced by TNF (enhanced permeability and release of protein S) are mediated by a pertussis toxin-sensitive G protein. In contrast, when other TNF-induced perturbations of endothelial cell function were examined in a similar fashion, a different result was observed (Fig. 7). The effect of TNF to promote the synthesis and expression of tissue factor procoagulant activity (22, 34) is not mediated by pertussis toxin (Fig. 7 *A*). Similarly, pertussis toxin did not alter TNF-mediated suppression of endothelial cell thrombomodulin (Fig. 7 *B*). Taken together, these results suggest that at least two separate transduction pathways are utilized by TNF that can be distinguished by their sensitivities to pertussis toxin.

Discussion

The results presented herein demonstrate that TNF can increase the permeability of cultured bovine aortic endothelial cell monolayers to macromolecules and smaller solutes. Although endothelial cell monolayers that form on the artificial surface used in these studies and by other laboratories (35–41) are more permeable than the physiologic endothelial monolayer within the vascular wall, they have provided a useful model to examine components of the response to TNF. As a result, our data indicate that the loss of intravascular solutes in the septic state may result from the direct action of TNF on endothelium to perturb its normal barrier function. Future studies that evaluate these in vitro findings within the context of the diverse biological effects of TNF in an in vivo model ultimately will be necessary to interpret the role of this TNF-induced perturbation in the pathogenesis of endotoxic shock.

The observations resulting from our studies indicate that TNF induces a progressive and marked change in disposition of the actin-based cytoskeleton that is accompanied by cell retraction (Fig. 1). These perturbations appear to result in the forma-

tion of inter-endothelial cell spaces (Fig. 1). Although a contractile event may be inferred, these observations do not yet permit us to assign a causal relationship between these coincident events. It is apparent, however, that the gaps between cells resulting from cell retraction are a route for passage of macromolecules across the monolayer. In support of the hypothesis that retraction of lateral cell margins, with its resultant enhanced permeability, may be brought about by an active cytoskeletal event, inhibition of the TNF-induced perturbations of cytoskeletal assembly by pertussis toxin is associated with attenuation of the action of TNF to augment endothelial permeability. While these observations do not rule out the possibility that a receptor-mediated transcytotic pathway results in at least a component of the enhanced permeability of endothelial monolayers in response to TNF (42–44), they do indicate that the para-cellular pathway via inter-endothelial cell gaps is the predominant avenue of solute exit.

Although these structural changes in endothelium after exposure to TNF were observed with a variety of bovine aortic endothelial cell cultures (from over a dozen animals and cells from passages 2–20), the cellular response may well be different when endothelium from different vascular beds are examined. For example, the shape and cytoskeletal changes depicted in human umbilical vein endothelial cells after longer (72–96 h) exposure to lower concentrations of TNF (45) differ from those shown in Fig. 1; this difference could be related to conditions of dosage, or it might be due to site or species differences. Experiments with multiple concentrations of TNF using endothelial cells derived from several locations (and in different species) will be required to resolve this issue.

Retraction of endothelial cells with the formation of gaps in the monolayer has been observed in response to vasoactive mediators such as histamine and serotonin (35, 46–50). The effect of histamine to activate endothelial inositol phospholipid metabolism and thereby mobilize intracellular calcium through an H_1 receptor-dependent mechanism is believed to underlie this physiologic response (49, 50). Despite certain similarities of the endothelial cell response to histamine and TNF, in opening a paracellular pathway of transudation, preliminary studies in our laboratory do not provide any evidence that TNF activates a similar rapid cellular response mechanism. Mechanisms of intracellular signal transduction after stimulation of cells with TNF have been difficult to identify. By focussing on early perturbations of endothelial cell physiology, such as protein S release and alteration in the actin-based cytoskeleton, we have found that one component of the endothelial cell response to TNF involves a pertussis toxin-sensitive regulatory G protein. Clark et al. (7) have proposed that the very rapid (5–10 min) retraction and increase in permeability of endothelial cells of the CPAE cell line induced by TNF is coupled by a pertussis toxin-sensitive dependent G protein and results from enhanced phospholipase A_2 activity and eicosanoid release. Furthermore, they found that cell shape and permeability change could be prevented by pretreatment with the inhibitor of eicosanoid synthesis BW755c, and by inhibitors of protein synthesis. In our hands, exposure of endothelium to TNF did not lead to the release of [^{3}H]arachidonic acid metabolites and the phospholipase A_2-activating protein inhibitor BW755c had no protective effect on TNF-induced perturbation of cytoskeleton/cell shape or barrier function over a wide range of concentrations and incubation times (data not shown). In addition, inhibitors of protein biosynthesis (which would have prevented synthesis of phospholipase

A_2-activating protein) had no effect on modulation of barrier function (Table I). The reason for the differences between our observations and those of Clark et al. (7) are not clear, however, it may be that their use of super-saturating concentrations of TNF (1,700 ng/ml) and of the CPAE cell line, as compared with our lower doses of TNF (doses that correlate with receptor occupancy) and use of bovine aortic endothelial cells passages 2–6, might explain why different mechanisms appear to be involved in the cellular response to TNF. On the other hand, our studies confirm and extend recent evidence for a critical role for a pertussis toxin-sensitive G protein in at least some biological responses to TNF (51).

In contrast to the protective effect of pertussis toxin on monolayer barrier function (Fig. 6), TNF-induced enhancement of endothelial cell tissue factor, which involves de novo transcription and translation (22, 34), and TNF-induced suppression of thrombomodulin activity, which involves a decline in the level of thrombomodulin antigen (52), were not affected by pertussis toxin (Fig. 7). These data suggest a model in which certain rapid cellular responses to TNF are mediated by a pertussis toxin-sensitive G protein, whereas other slower perturbations of cellular physiology, including some that involve regulation at the transcriptional/translational level, are brought about by recruitment of different intracellular mechanisms.

Summary

Endothelium is an important target of tumor necrosis factor/cachectin (TNF), a central mediator of the host response in endotoxemia and Gram-negative sepsis. In this report, TNF is shown to increase the permeability of endothelial cell monolayers to macromolecules and lower molecular weight solutes by a mechanism involving a pertussis toxin-sensitive regulatory G protein. Within 1–3 h of exposure to TNF (5 nM), changes in cell shape/cytoskeleton occurred that led to disruption of monolayer continuity with the formation of intercellular gaps. Correlated with these structural changes was an increase in endothelial permeability to macromolecular and lower molecular weight tracers; time-dependent, reversible increases in passage of these tracers, evident by 1–3 h, were observed after addition of TNF to cultures. Perturbation of barrier function by TNF also depended on the dose of TNF added being half-maximal by ~0.4 nM. Only a brief exposure (15 min) of TNF to endothelium was required to induce an increase in permeability, and this was not prevented by the presence of cycloheximide or actinomycin D. Preincubation of monolayers with pertussis toxin blocked in parallel TNF-induced increased passage of solutes and cell shape/cytoskeletal perturbation, indicating the close correlation between these changes in endothelial cell function. In contrast, pertussis toxin did not alter TNF-induced modulation of two endothelial cell coagulant properties. These data provide evidence for two intracellular pathways of TNF action that are distinguishable by pertussis toxin and provide insight into a mechanism underlying loss of solute from the intravascular space mediated by TNF: alteration in endothelial cell barrier function.

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