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Research Article

The neutrophil has been implicated as an important mediator of vascular injury, especially after endotoxemia. This study examines neutrophil-mediated injury to human microvascular endothelial cells in vitro. We found that neutrophils stimulated by formyl-methionyl-leucyl-phenylalanine (FMLP), the complement fragment C5a, or lipopolysaccharide (LPS) (1-1,000 ng/ml) alone produced minimal endothelial injury over a 4-h assay. In contrast, neutrophils incubated with endothelial cells in the presence of low concentrations of LPS (1-10 ng/ml) could then be stimulated by FMLP or C5a to produce marked endothelial injury. Injury was maximal at concentrations of 100 ng/ml LPS and 10(-7) M FMLP. Pretreatment of neutrophils with LPS resulted in a similar degree of injury, suggesting that LPS effects were largely on the neutrophil. Endothelial cell injury produced by LPS-exposed, FMLP-stimulated neutrophils had a time course similar to that induced by the addition of purified human neutrophil elastase, and different from that induced by hydrogen peroxide (H2O2). Further, neutrophil-mediated injury was not inhibited by scavengers of a variety of oxygen radical species, and occurred with neutrophils from a patient with chronic granulomatous disease, which produced no H2O2. In contrast, the specific serine elastase inhibitor methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone inhibited 63% of the neutrophil-mediated injury and 64% of the neutrophil elastase-induced injury. However, neutrophil-mediated injury was not inhibited significantly by 50% serum, 50% plasma, or purified alpha 1 proteinase inhibitor. […]

Neutrophil-mediated Injury to Endothelial Cells

Enhancement by Endotoxin and Essential Role of Neutrophil Elastase

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Abstract

The neutrophil has been implicated as an important mediator of vascular injury, especially after endotoxemia. This study examines neutrophil-mediated injury to human microvascular endothelial cells in vitro. We found that neutrophils stimulated by formyl-methionyl-leucyl-phenylalanine (FMLP), the complement fragment C5a, or lipopolysaccharide (LPS) (1–1,000 ng/ml) alone produced minimal endothelial injury over a 4-h assay. In contrast, neutrophils incubated with endothelial cells in the presence of low concentrations of LPS (1–10 ng/ml) could then be stimulated by FMLP or C5a to produce marked endothelial injury. Injury was maximal at concentrations of 100 ng/ml LPS and 10^{-7} M FMLP. Pretreatment of neutrophils with LPS resulted in a similar degree of injury, suggesting that LPS effects were largely on the neutrophil. Endothelial cell injury produced by LPS-exposed, FMLP-stimulated neutrophils had a time course similar to that induced by the addition of purified human neutrophil elastase, and different from that induced by hydrogen peroxide (H_2O_2). Further, neutrophil-mediated injury was not inhibited by scavengers of a variety of oxygen radical species, and occurred with neutrophils from a patient with chronic granulomatous disease, which produced no H_2O_2. In contrast, the specific serine elastase inhibitor methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone inhibited 63% of the neutrophil-mediated injury and 64% of the neutrophil elastase-induced injury. However, neutrophil-mediated injury was not inhibited significantly by 50% serum, 50% plasma, or purified α_1 proteinase inhibitor. These results suggest that, in this system, chemotactic factor-stimulated human neutrophil injury of microvascular endothelial cells is enhanced by small amounts of LPS and may be mediated in large part by the action of neutrophil elastase.

Introduction

Bacterial endotoxins have been implicated in the pathogenesis of a wide variety of human and animal disorders (1). In particular, endotoxemia appears to result in injury to a number of crucial organ systems, especially lung and kidney. With regard to the lung, the adult respiratory distress syndrome (ARDS)[1] may occur in conjunction with endotoxemia, and is associated with severe injury to the gas-exchange apparatus (2). In human beings, where neutrophils can be recovered from broncho-alveolar lavage in early stages (3), and in animals, where infusion of endotoxin produces lung injury (4) that is abolished by neutrophil depletion (5), the neutrophil has been suggested to play a central role in the pathogenesis of endotoxin-associated lung injury (6). The neutrophil has also been implicated in the pathogenesis of the generalized Schwartzman reaction, in which renal cortical necrosis and severe vascular injury have been shown after two sequential endotoxin injections. This type of vascular injury response was also abolished by prior depletion of circulating neutrophils (7).

The mechanisms by which endotoxin promotes neutrophil-mediated vascular injury are unclear, since endotoxin has many actions (8). Early interest centered on the ability of the lipopolysaccharide (LPS) fraction of endotoxin (which possesses most of the biologic activity) (9) to activate complement intravascularly (10). However, although intravascular generation of potent complement-derived chemotactic factors may be important, a considerable body of data suggests that complement activation by itself is insufficient to produce severe lung or kidney injury (11, 12).

In this regard, we have recently shown that even small amounts of LPS may serve to enhance both the stimulation of neutrophils in vitro and neutrophil-mediated injury in vivo. Thus, in vitro, LPS at concentrations of 1–10 ng/ml "primes" neutrophils such that they respond to chemotactic factors with an enhanced release of toxic oxygen radicals (13) and lysosomal enzymes (14). LPS has also been shown directly to stimulate neutrophil adherence to plastic (15), and in our studies, to human endothelial cells.[2] In vivo, we have found that the simultaneous infusion of both chemotactic factors and trace amounts of LPS (100 ng) in rabbits enhanced neutrophil sequestration within the lung, at least partly reflecting neutrophil adherence. In addition, the infusion of chemotactic factors and LPS led to a marked enhancement of neutrophil-mediated injury to lung endothelium, which was associated with increased vascular permeability (submitted for publication).

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1. *Abbreviations used in this paper:* AAPVCK, methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone; ARDS, adult respiratory distress syndrome; C5a, chemotactic fragment of the fifth component of complement; DMSO, dimethyl sulfoxide; FMLP, formyl-methionyl-leucyl-phenylalanine; ^{111}In, ^{111}indium-tropolonate; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; α_1 PI, α_1 proteinase inhibitor; PMA, phorbyl myristate acetate; SOD, superoxide dismutase; TLCK, *N*-alpha-tosyl-L-lysine-chloromethyl ketone.

2. Unpublished observations that show LPS increases neutrophil adherence in a dose-dependent manner to human endothelial cells.

Just as the mechanisms by which LPS exerts its effects are unclear, so are the mechanisms by which neutrophils injure tissues. Both oxygen radicals (16–18) and neutrophil-derived proteases (19, 20) have been implicated in neutrophil-mediated injury to the endothelium in vivo, in isolated perfused organs, and to cultured endothelial cells in vitro.

Accordingly, the goals of this study were twofold. First, we sought to examine the interaction of LPS with stimulated human neutrophils and cultured human microvascular endothelial cells, to test the hypothesis that LPS would enhance neutrophil-mediated injury stimulated by chemotactic factors. Second, these experiments permitted us to begin to investigate the mechanisms by which neutrophils injure human endothelial cells.

We show that LPS in low concentrations (1–10 ng/ml) was capable of enhancing stimulated neutrophil-mediated injury to endothelial cells. Further, we demonstrate that neutrophil elastase was the major identifiable injurious agent. These data may be relevant to LPS-induced vascular injury in vivo.

Methods

Reagents. All reagents and plasticware used in this assay were tested before use for the presence of LPS using the Limulus Amebocyte Lysate kit from Associates of Cape Cod, Inc. (Woods Hole, MA). This procedure detects as little as 0.01 ng LPS/ml. Sterile plastics were free of detectable LPS contamination, and all reagents, tested at the concentration used in the injury assay, contained <0.1 ng/ml LPS with the exception of human neutrophil elastase, which had 1 ng LPS/0.1 U of elastase.

The assay buffer employed was Kreb's-Ringer phosphate buffer, pH 7.2, with 0.2% dextrose (5% dextrose in 0.2% sodium chloride, injectable, Abbott Laboratories, North Chicago, IL) and 0.5% human serum albumin (prepared without preservatives or detectable LPS levels in a 1% solution by Cutter Laboratories, Emeryville, CA). The salts comprising the buffer were purchased from Mallinkrodt (Paris, KY), and had undetectable LPS levels. All components were freshly diluted with LPS-free saline (0.9% saline for irrigation, Abbott Laboratories) on each experimental day.

LPS was prepared by the method of McIntire (21) from *Escherichia coli* K235 and was a kind gift of Dr. David Morrison of Emory University, Atlanta, GA. The lyophilized LPS was dissolved in LPS-free saline at 500 μg/ml. Frozen aliquots were thawed and sonicated using a Sonicator Cell Disrupter (Ultrasonics, Inc., Plainview, NY) with a microtip at an amplitude setting of 2 for two 10-s periods before being diluted in assay buffer.

The biologically active chemotactic fragment of the fifth component of complement (C5a) was purified from human serum by a procedure modified from that of Fernandez and Hugli (22), as reported previously (23). The chemotactic factor formyl-methionyl-leucyl-phenylalanine (FMLP, Vega Biochemicals, Tucson, AZ) was kept frozen in dimethyl sulfoxide (DMSO, Fisher Scientific Co., Fair Lawn, NJ) at 10^{-3} M and diluted in assay buffer.

Human neutrophil elastase was purified according to the method of Baugh and Travis (24). The procedure involved affinity chromatography of crude extracts of normal human neutrophil leukocyte granules using bovine lung trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) bound to sepharose 4B (Pharmacia Fine Chemicals, Upsalla, Sweden). 1 U of elastase was defined as the amount of elastase that solubilized 1 μg of insoluble bovine ligamentum nuchae elastin in 1 h at 37°C.

The specific elastase inhibitor methoxy-succinyl-alanyl-alanyl-prolyl-valyl-chloromethyl ketone (AAPVCK) was custom synthesized by Bachem, Inc. (Torrance, CA) (25). A trypsin and papain inhibitor, *N*-alpha-tosyl-L-lysine-chloromethyl ketone (TLCK), was purchased from Vega Biochemicals. Both the AAPVCK and TLCK were dissolved in DMSO, then diluted to 10^{-4} M in assay buffer (0.05% DMSO, final concentration). Human α_1 proteinase inhibitor (α_1 PI) was purchased from Sigma Chemical Co. and purified by affinity chromatography on Cibacron Blue FG3-A (Pharmacia Fine Chemicals) to remove albumin (26). After dialysis there was a single band on gel electrophoresis. Other reagents added to the assay were superoxide dismutase (SOD, Diagnostic Data, Inc., Mountain View, VA), catalase (17,600 U/mg, Sigma Chemical Co.), sodium azide (Sigma Chemical Co.), thiourea (Sigma Chemical Co.), and hydrogen peroxide (H_2O_2, Fisher Scientific Co.). Activity of H_2O_2 was determined by its capacity to oxidize scopoletin (27).

Endothelial cells. Human microvascular endothelial cells were isolated from fresh, healthy adipose tissue as described by Kern et al. (28). Briefly, the tissue was minced, collagenase digested, and the suspension was sieved through nylon mesh and layered onto a 5% albumin gradient. This procedure separated large endothelial clumps from fibroblasts and adipocytes that were usually present as single cells. The yield was essentially pure endothelial cells, which were plated into 35-mm diameter wells (Costar, Cambridge, MA). The growth medium was MCDB 131, which contains 10 ng/ml epidermal growth factor (Sigma Chemical Co.), 1 μg/ml hydrocortisone (Sigma Chemical Co.), and 2% fetal bovine serum (Irvine Scientific, Santa Anna, CA), and is a selective medium for endothelial cells (29). The monolayers demonstrated the typical cobblestone morphology of endothelial cells. In addition, the cells expressed Factor VIII antigen and angiotensin converting enzyme activity and demonstrated uptake of acetylated low density lipoprotein (28).

Human umbilical vein endothelial cells were harvested by collagenase digestion according to the method of Gimbrone et al. (30) and grown as previously described (31).

Neutrophils. Human blood neutrophils were prepared using plasma-Percoll gradients and avoiding LPS exposure, by a method we have previously described (14). In brief, whole citrated blood was centrifuged, the packed cells sedimented through 6% dextran, and the mixed leukocytes then separated through 42 and 51% plasma-Percoll gradients. The resulting neutrophil preparation was 95% pure and 99% viable based on trypan blue exclusion. Neutrophils isolated by this procedure showed enhanced chemotactic responsiveness, less spontaneous change of shape, and lower baseline secretion of superoxide anion and lysosomal enzymes than did neutrophils prepared by the traditional Ficoll-Hypaque method (14). The effects of Ficoll-Hypaque preparation on neutrophil behavior in vitro could be duplicated in the plasma-Percoll cells by the addition of small amounts of LPS (14).

Injury assay. The assay used was a modification of previously described monolayer injury assays (18, 20). The primary cultures of endothelial cells were passed by trypsinization into 1-cm^2 assay wells (Costar) and maintained 2–3 d postconfluence ($1–2 \times 10^5$ cells/well). On the day of assay, the endothelial cells were washed twice with assay buffer. To label the monolayers, ^{111}indium-tropolonate (^{111}In) (72 mCi/ml, New England Nuclear, Boston, MA) at 10 μCi/ml was first incubated with 4×10^{-3} M tropolone (Sigma Chemical Co.) in assay buffer for 1 min, and this solution was then added to the cells to achieve a final concentration of 4×10^{-4} M tropolone and 1 μCi of ^{111}In per 10^5 cells. The monolayers were incubated for 15 min at 25°C, washed three times with assay buffer, and used immediately. The cells incorporated ~10% of the added label to give an average of 177,000 cpm/10^5 cells.

Neutrophils, suspended in assay buffer, were added to the endothelial monolayers at a final concentration of 1×10^6/well (10 neutrophils to 1 endothelial cell). LPS with or without other inhibitors were added at this time to triplicate sample wells. In other wells, neutrophil elastase or H_2O_2 was added in the absence of neutrophils. The assay was incubated for 1 h at 37°C in a humidified, 5% CO_2 incubator. The chemotactic factors FMLP or C5a were then added to the appropriate wells, and the incubation was continued for an additional 3 h. To determine spontaneous release for each experiment, triplicate control wells containing 1.1 ml (the final fluid volume of all the reaction mixtures) of assay buffer were incubated in parallel for the 4-h period. At each hour, 100 μl was carefully removed from all wells and placed in tubes to be counted in a Beckman 8000 gamma counter. After 4 h of incubation, the wells were gently washed three times with assay buffer. The wash fluids from each well were pooled, centrifuged at 250 *g* for 6 min, and the supernatant and pellet were separated for counting. Cumulative ^{111}In release over time was calculated by correcting each hourly aliquot for the total well

volume at that time. In contrast, total cpm released was determined by adding the cpm in each aliquot to that in the washes at the end of the experiment that might include loosely adherent radioactivity released by the washes. Releasable cpm for each experiment were determined by subjecting triplicate monolayers to two freeze-thaw cycles and washing as described above (an average of 71.9±4.7% [SEM] of the incorporated radioactivity was released). If $A_{(1-4)}$ = cpm in each 100-μl sample from hours 1–4, B = cpm in supernatant from washes, C = cpm in pellet from washes, and T = cpm releasable by freeze-thaw, then percent ^{111}In release at each time point was calculated as: ($A \times$ well volume correction)/(T) $\times$ 100, and the total percent ^{111}In release was calculated as: ($A_{(1-4)} + B + C$)/(T) $\times$ 100. Results displaying total percent ^{111}In release include the percentage of counts present in the pellet from each well washing as calculated by (C)/(T) $\times$ 100. Percent inhibition was determined by the formula: 1 − (% release with inhibitor − spontaneous release)/(% release without inhibitor − spontaneous release) $\times$ 100.

In three experiments, we compared the release of ^{111}In to the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) (32) from endothelial monolayers. Monolayers were subjected to 1, 2, or 3 freeze-thaw cycles, the wells were washed, and LDH or ^{111}In was measured from the supernatant only. ^{111}In and LDH release correlated well (correlation coefficient of 0.84).

Release of O_2^- by neutrophils. Release of O_2^- from neutrophils was determined as described by Johnston et al. (33) using SOD-inhibitable reduction of cytochrome *c*. Neutrophils (2.5×10^6) were preincubated for 1 h at 37°C in suspension with assay buffer or 1 μg/ml LPS (plus, when appropriate, SOD or AAPVCK). The stimulus FMLP was then added along with cytochrome *c*, and the reaction mixture was incubated for 10 min at 37°C.

Electron microscopy. Pellet material from several wells of endothelial cells that had been incubated with LPS/FMLP-stimulated neutrophils was collected, fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in osmium, and dehydrated in ethanol. Thin sections were poststained with uranyl acetate and Reynold's lead and viewed on a Philips 400 T electron microscope.

Statistics. The effect of differing treatments on total ^{111}In release from endothelial cell monolayers was compared by one-way analysis of variance and the Tukey multiple comparisons test, calculated using a proprietary statistical package (Statistical Analysis System, Gary, NC). Comparisons between the time course of release under various conditions were made employing a Bonferroni multiple comparison test analyzing the log of the differences between different groups. This latter analysis was performed on another proprietary statistical package (BMDP, Inc., Los Angeles, CA). Groups were considered different if the P value was ≤0.05.

Results

Effect of LPS on neutrophil-mediated endothelial injury. The protocol of these experiments was based on the previously discussed ability of LPS to increase neutrophil adhesiveness and to prime neutrophils for an enhanced response to chemotactic factor stimulation. Endothelial cells and neutrophils were incubated together with LPS for 1 h before the addition of the chemotactic factors to stimulate neutrophil secretion of enzymes and oxygen metabolites.

As shown in Fig. 1, the incubation of LPS with neutrophils and endothelial cells and subsequent addition of a high dose of the stimulus FMLP (10^{-6} M) caused substantial endothelial injury after 4 h, as assessed by ^{111}In release. Neutrophils stimulated with the same concentration of either FMLP or LPS alone did not elicit significant release of ^{111}In from endothelial monolayers. LPS and FMLP in the absence of neutrophils had no injurious effect, thus indicating that neutrophils played a necessary role.

The percent of label present in the pellet (obtained by centrifugation of the wash buffers at the end of the incubation) from each test condition is also depicted in Fig. 1. It should be noted that pellet counts were extremely low, indicating minimal detachment of intact endothelial cells. ^{111}In cpm in the pellet from buffer control wells was 2.1±0.4% of the total releasable cpm, whereas in wells containing neutrophils plus LPS and FMLP it comprised 4.7±0.7%. Pelletable counts thus remained a small and relatively constant percentage of the radioactivity released into the medium by the various treatments, ranging from 16.4 to 18.6% of the cpm actually released in all samples.

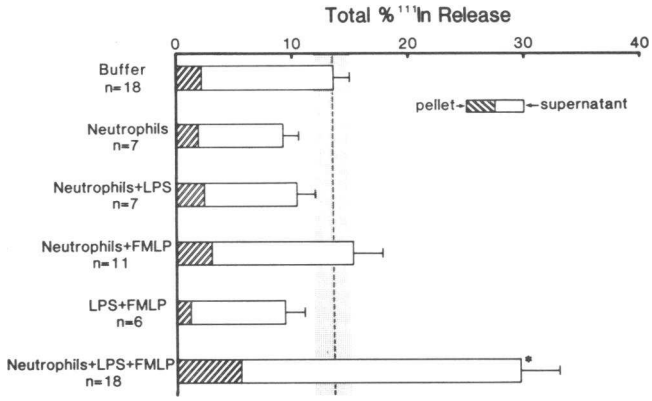

Figure 1. The effect of LPS and FMLP on neutrophil-mediated endothelial cell injury. Neutrophils in the presence or absence of 1 μg/ml LPS were incubated for 1 h with endothelial cells (10 neutrophils to 1 endothelial cell) before the addition of 10^{-6} M FMLP to the appropriate wells. After an additional 3 h of incubation, the wells were washed and the washes separated into pellet and supernatant and counted. Control wells containing buffer were incubated in parallel for 4 h. Data is depicted as the mean±SEM of the number of experiments indicated. The shaded area represents the mean±1 SEM of the control release. Neutrophils + LPS + FMLP caused significant injury (P = 0.003) (*).

The pellet from wells incubated with neutrophils, LPS, and FMLP was examined by both light and electron microscopy. After the low-speed centrifugation described in the assay protocol, the pellet contained many neutrophils, considerable debris, and very few whole endothelial cells. Using trypan blue exclusion as a measure of viability, the neutrophils were able to exclude trypan blue while 90% of the endothelial cells took up trypan blue. Electron microscopy revealed neutrophils and disrupted endothelial cells in the pellet, as shown in Fig. 2. Taken together, these data suggest that, in this system, ^{111}In release was a consequence of endothelial cell lysis rather than detachment.

During endotoxemia, and thus after interaction with blood components, concentrations of circulating LPS have been measured to be between 0.5 and 5 ng/ml (34), figures that likely represent an underestimate of the amount of LPS available for transfer to membranes. We therefore investigated the concentration of LPS necessary to promote neutrophil-mediated injury using 10^{-6} M FMLP as the stimulus. As shown in Fig. 3, an effect on ^{111}In release was seen at LPS concentrations as low as 1–10 ng/ml, and the injury reached a maximum at concentrations of 100–1,000 ng/ml. In most subsequent studies, in order to enhance the signal-to-noise ratio, we employed a maximal LPS concentration of 1 μg/ml.

The effect of different concentrations of FMLP was also studied and results are shown in Fig. 4. Measurable injury was

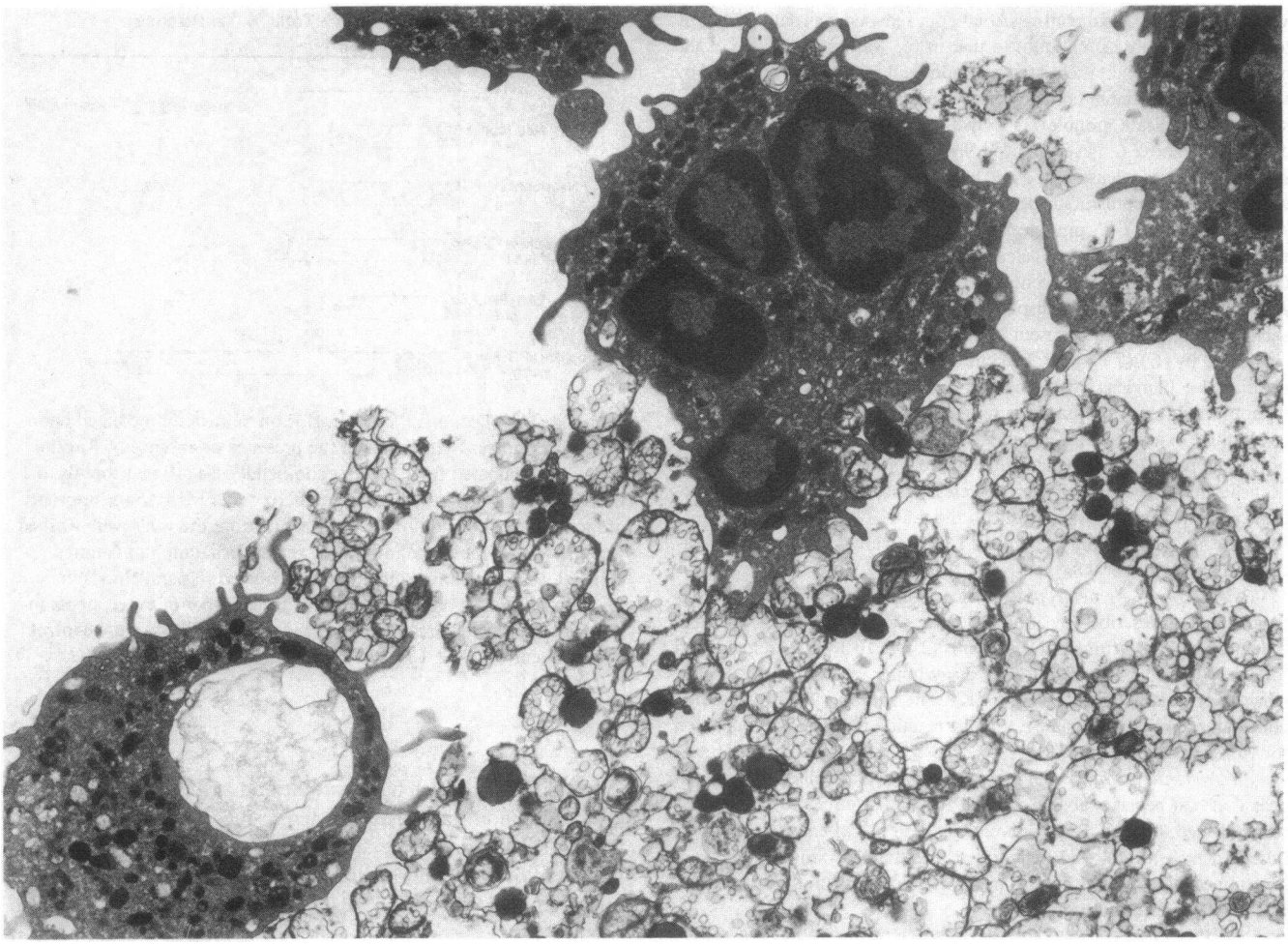

Figure 2. Transmission electron micrograph of the pelletable material from injured endothelial monolayers. The endothelial cells were incubated with 10^6 neutrophils and 1 μg/ml LPS for 1 h, 10^{-6} M FMLP was added, and the incubation continued for a total of 4 h. The wells were washed and the pellets from these washings were fixed and processed as described. An apparently intact neutrophil is shown surrounded by endothelial cell debris. × 9,200.

seen at 10^{-8} M and a maximum effect was achieved at concentrations of 10^{-7} M or higher. The biologically relevant complement component C5a was tested for its capacity to stimulate neutrophil-mediated injury. Incubation of neutrophils with 10^{-7} M C5a and 1 μg/ml LPS resulted in the same magnitude of endothelial injury as that seen using 10^{-7} M FMLP and LPS (^{111}In release was 28.7±4.7% with neutrophils incubated with C5a and LPS, compared with 13.0±1.2% for neutrophils and C5a alone, n = 4).

Pretreatment studies. LPS has been shown to affect both neutrophils (13–15)[2] and some types of endothelial cells (20). In an attempt to determine whether the effect of LPS was to increase the injurious potential of the neutrophil, or to increase the susceptibility of the endothelial cell, neutrophils and endothelial cells were separately pretreated with LPS before assaying injury. The results are shown in Fig. 5. Neutrophils were exposed to 100 ng/ml LPS for 1 h, washed, and then added to the endothelial cells with FMLP. This procedure produced nearly the same magnitude of injury seen with co-incubation of neutrophils and LPS with endothelial cells. In contrast, pretreatment of endothelial cells with LPS (up to 1 μg/ml) for 1 h before washing and the addition of neutrophils and FMLP resulted in little ^{111}In release above baseline. It is not known how much LPS adhered to the surface of the endothelial cells and the culture well after washing. It is possible, therefore, that some residual LPS was available to act on the neutrophils. Conversely, LPS itself may have had a small direct effect on the endothelial cells. Neutrophils pretreated with LPS and then added to the endothelial cells with buffer alone also produced a small increase in ^{111}In release. Under these circumstances, the centrifugation of the neutrophils after LPS priming may have caused some activation. In agreement with our other data, endothelial cells pretreated with LPS before the addition of neutrophils, without stimulus, released no more ^{111}In than buffer controls. From these data, it appears that, in this system, LPS acts primarily on the neutrophil to enhance neutrophil-mediated injury.

Time course of injury: effects of neutrophils, elastase, and H_2O_2. Injury to endothelial cells was followed over the 4-h assay and the results are shown in Fig. 6. ^{111}In release is depicted as the cumulative value at each time point by counting an aliquot and correcting for the well volume. Although there was some label present in the supernatant at the start of the incubation, as shown by the release obtained in the presence of buffer alone, the endothelial cells released almost no additional label spon-

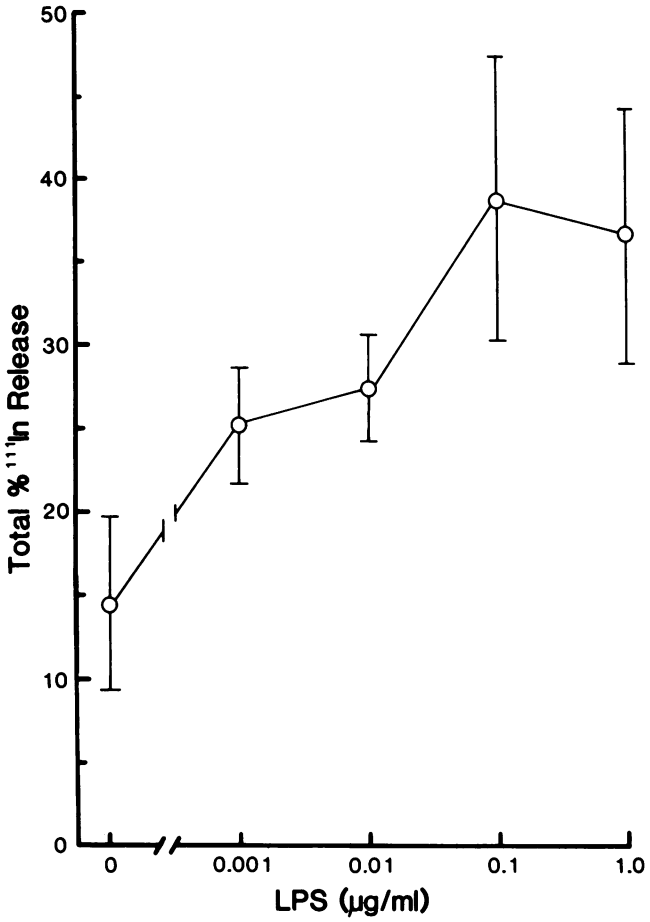

Figure 3. The effect of varying concentrations of LPS on release of ^{111}In from cultured endothelial cells. Neutrophils and endothelial cells were incubated with each concentration of LPS for 1 h before the addition of 10^{-6} M FMLP. After a total of 4 h of incubation, the wells were washed and the total percent ^{111}In release was calculated. The points represent the means and the bars the SD of triplicate wells in two experiments.

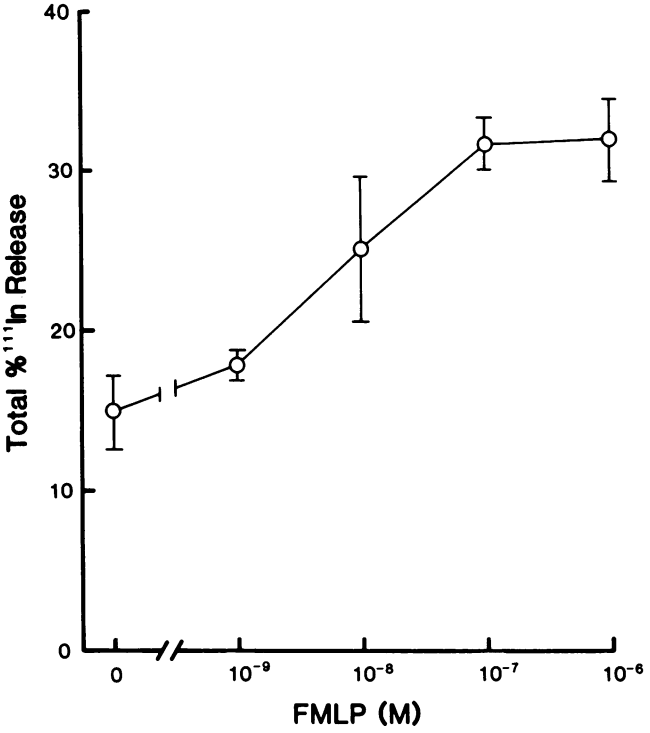

Figure 4. Injury response to varying concentrations of FMLP. Endothelial cells and neutrophils were incubated with 1 μg/ml LPS for 1 h before the addition of FMLP. After an additional 3 h of incubation the wells were washed and the total % ^{111}In release was calculated. Points represent the means and the bars the SD of three determinations.

taneously during the next 3 h of incubation. Spontaneous release of ^{111}In was calculated to be 0.05%/h during the 0–3-h incubation period. When neutrophils were incubated with endothelial cells and LPS and then stimulated with FMLP, there was significant (P = 0.003) injury within 1 h after the addition of FMLP, and the magnitude of this injury increased substantially over the next 2 h. The rate of release of label was calculated to be 3.5%/h, greatly enhanced when compared with release from buffer-containing wells.

To compare neutrophil-mediated injury with injury caused by defined mediators that might be produced by stimulated neutrophils, H_2O_2 and purified neutrophil elastase were added to endothelial monolayers as agents that might cause oxidant and proteolytic injury, respectively. The reagents were added at time zero and incubated for 4 h following the same protocol as for the neutrophil injury system. Purified human neutrophil elastase at 0.5 U/ml (an amount calculated to equal ~10% of the total neutrophil elastase present in 10^6 neutrophils) (35) was able to elicit lytic injury with a time course similar to that of neutrophil-mediated injury (Fig. 6). Including the washes at the end of the experiment, the total ^{111}In release from elastase-treated endothelial cells was 30.9±3.4%, with 4.8±2.0% present in the pellet (three experiments). Thus, this concentration of neutrophil elastase caused endothelial cell lysis of the same time course and magnitude as that produced by LPS/FMLP-stimulated neutro-

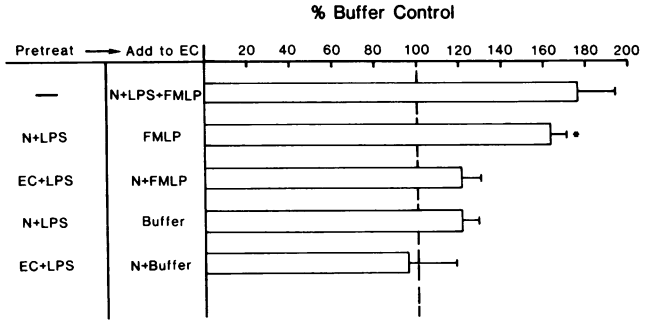

Figure 5. The effect of pretreatment of neutrophils or endothelial cells with LPS. Endothelial cells were incubated with LPS at 100 ng/ml for 1 h at 37°C, the monolayers were washed twice, and neutrophils were added with buffer or 10^{-6} M FMLP. Neutrophils were pretreated with 100 ng/ml LPS for 30 min at 37°C, washed once, and incubated at room temperature for a total pretreatment time of 1 h. With either pretreatment, neutrophils and endothelial cells were mixed with buffer or 10^{-6} FMLP and the wells were incubated an additional 3 h. Data are depicted as the mean±SEM of three experiments. Pretreatment of neutrophils with LPS before adding FMLP caused significantly greater injury than pretreatment of endothelial cells.(*) (P = 0.02). Mean±SEM ^{111}In release for buffer controls was 11.3±7.4%.

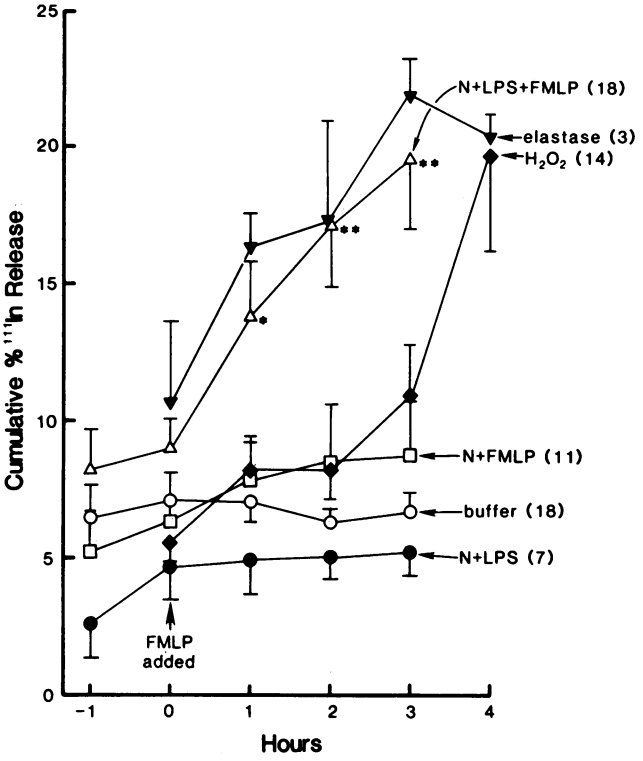

Figure 6. The time course of ^{111}In release from endothelial monolayers injured by stimulated neutrophils, H_2O_2, or elastase. At −1 h, neutrophils and 1 μg/ml LPS were added to endothelial monolayers. At zero time, 10^{-6} M FMLP was added to the appropriate wells and the incubation was continued for a total of 4 h. Elastase (0.5 U/ml) or H_2O_2 (10^{-4} M) was added to endothelial cells and incubated for 4 h. At each hour, 100 μl was removed from each well and counted and the cumulative ^{111}In release was calculated. The points represent the mean and the bars the SEM; the number of experiments is indicated in parentheses. Compared with FMLP-stimulated neutrophil controls, neutrophils + LPS + FMLP caused significant release at 1 h(*) ($P = 0.003$), and the magnitude of the injury increased over the second and third h(**) ($P = 0.0001$).

phils without inducing detachment of cells (i.e., increasing counts in the pellet). Higher concentrations of elastase caused ^{111}In release earlier, and endothelial cells exposed to 4 U/ml elastase were observed to have become "round" but not detached by the end of the incubation period.

Hydrogen peroxide at 10^{-4} M elicited comparable total ^{111}In release to that produced by neutrophils sequentially stimulated with LPS and FMLP. This concentration of H_2O_2 is approximately fivefold greater than that measured in vitro after stimulation of neutrophils with LPS and FMLP (13), but was selected on the assumption that local levels of H_2O_2 at the cell surface would be considerably higher than amounts in the supernatant. As can be seen by reference to the time course in Fig. 6, although H_2O_2 produced a similar amount of label release from the endothelial cells, the time course differed from that of neutrophil-mediated damage. In contrast to stimulated neutrophils or elastase, H_2O_2 did not cause significant label release into the aliquots until the fourth h of incubation. However, the total label release at the end of the fourth hour, which includes washes that might release loosely adherent counts, was 58.9±10.9%, with 8.3±2.6% present in the pellet.

Human umbilical vein endothelial cells, which have been commonly used in endothelial injury systems, were also employed for these studies. In experiments comparing microvascular with umbilical vein endothelial cells, the time course and magnitude of neutrophil-mediated injury was similar for the two cell types, although umbilical vein cells were able to be injured by neutrophils and FMLP alone (data not shown). The two kinds of endothelial cells responded quite differently to H_2O_2-induced injury, however. Hydrogen peroxide at 10^{-5} M elicited significant ^{111}In release from umbilical vein cells (196.4±8.3% of buffer controls over 4 h, $n = 4$), but this same concentration caused minimal injury to microvascular endothelial cells (119.5±11.8% of buffer ^{111}In release over 4 h, $n = 4$). Thus, umbilical vein endothelial cells may be more susceptible to oxidative injury than microvascular endothelial cells.

Mechanism of injury. Since the time course of neutrophil-mediated injury paralleled that due to elastase, but was quite different from that of injury induced by H_2O_2, we studied neutrophils from a patient with chronic granulomatous disease (36). In one experiment, performed in quadruplicate, these neutrophils, which were shown not to produce superoxide anion or H_2O_2, induced ^{111}In release from endothelial cells that was equivalent to that of normal neutrophils when both were incubated with LPS and stimulated with FMLP (chronic granulomatous disease neutrophils caused 144.4±21.2% ^{111}In release over buffer controls, compared with 144.7±19.7% ^{111}In release with normal neutrophils).

Inhibitor studies were begun to assess the relative roles of proteinases and oxygen radicals in neutrophil-mediated endothelial injury. All of the inhibitors were added at the time of the addition of neutrophils and LPS, i.e., 1 h before the addition of FMLP to the assay system. As seen in Fig. 7, endothelial injury was inhibited by 75% with a combination of inhibitors of oxygen

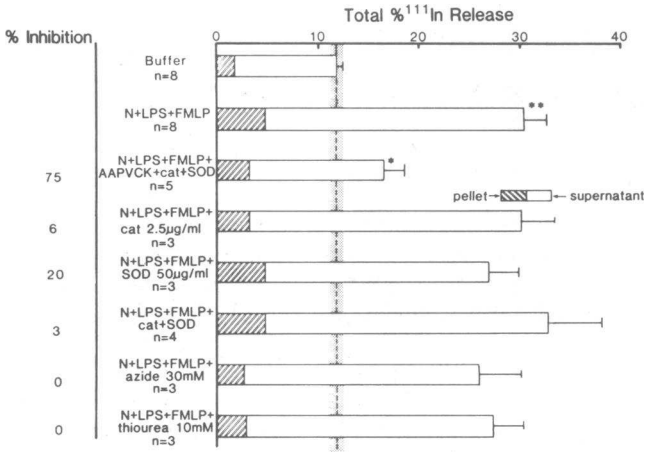

Figure 7. The effect of inhibitors of oxidative or proteolytic activity on endothelial injury. Neutrophils, 1 μg/ml LPS, and the inhibitors at the concentrations shown were incubated with endothelial monolayers for 1 h before the addition of 10^{-6} M FMLP. After an additional 3 h incubation, the wells were washed and the washes separated into pellet and supernatant and counted. Data is depicted as the mean±SEM of the number of experiments indicated. The shaded area represents the mean±1 SEM of the spontaneous release. The stimulated neutrophil-mediated injury was significant ($P = 0.0001$)(**), and the inhibition of injury by the combination of AAPVCK + catalase + SOD was significant at $P = 0.05$(*). No other inhibitors shown had a significant effect on injury. Percent inhibition was calculated by averaging the mean percent inhibition from each experiment.

species (catalase and SOD), and the specific elastase inhibitor AAPVCK. Catalase or SOD alone had no significant effect at either the dose shown or at a dose 100 times (catalase) or two times (SOD) more concentrated. Although they were ineffective at inhibiting neutrophil-mediated injury, both of these agents were tested in separate experiments and shown to be active against H_2O_2 or O_2^-, using specific assays for these radicals (33, 37). Catalase at 2.5 μg/ml inhibited endothelial injury produced by 10^{-4} M H_2O_2 by 95±4% (n = 4). SOD inhibited completely the ferricytochrome c reduction that results from O_2^- production by stimulated neutrophils (100%, n = 6). The combination of catalase and SOD also had no significant inhibitory effect on endothelial injury over the 4 h of the assay (Fig. 7). Thus, the combination of catalase and SOD did not reproduce the inhibition produced by catalase + SOD + AAPVCK. Since catalase and SOD are relatively large molecules, we considered it possible that they might not be present in high enough concentration at the neutrophil-endothelial interface. Accordingly, a smaller molecule that scavanges hydroxyl radicals, thiourea (37), was tested. Neither this agent nor azide, an inhibitor of myeloperoxidase (38), inhibited neutrophil-mediated injury (Fig. 7). In data not shown, DMSO, which also scavanges hydroxyl radicals (39), was unable to inhibit injury (% inhibition = 11%).

The effects of the specific elastase inhibitor AAPVCK and a related inhibitor, TLCK, are demonstrated in Fig. 8. At a concentration of 10^{-5} M, AAPVCK was nearly as effective (63% inhibition) as the combination of inhibitors (catalase + SOD + AAPVCK, 75% inhibition). Higher concentrations showed no additional inhibitory activity (data not shown). The related chloromethyl ketone derivative, TLCK, which inhibits trypsin and papain but lacks specificity for elastase, was added at the same concentration and caused slight, but statistically insignificant, inhibition. The combination of TLCK, catalase, and SOD had no significant inhibitory effect, in marked contrast to the combination of AAPVCK, catalase, and SOD. AAPVCK, but not TLCK (both at 10^{-5} M), was able to inhibit injury caused by purified neutrophil elastase (0.5 U/ml) to approximately the same degree (64.2±22.4%, n = 6) as it inhibited stimulated neutrophil-induced injury. Similarly, AAPVCK was able to inhibit injury in our single experiment with chronic granulomatous disease neutrophils to a similar degree (60%). AAPVCK (10^{-5} M) had no effect on LPS-primed, FMLP-stimulated neutrophil oxygen radical secretion, as assessed by superoxide release (n = 6). Similarly, we found that this concentration of AAPVCK did not inhibit neutrophil chemotaxis in vitro (manuscript in preparation). In addition, 10^{-5} M AAPVCK did not prevent H_2O_2-mediated endothelial cell injury when added at the same time as H_2O_2. These data, taken together, suggested that neutrophil elastase was an important mediator of endothelial injury in this system.

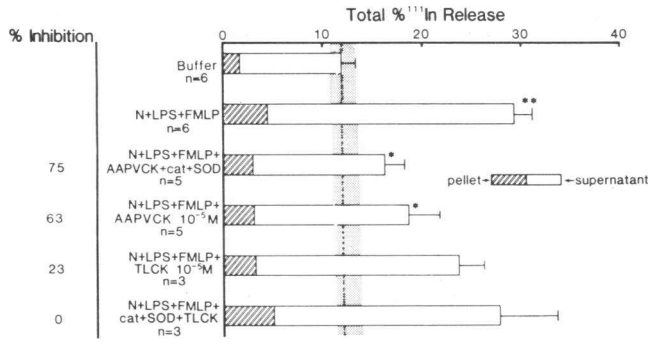

Figure 8. The effect of chloromethyl ketone inhibitors on inhibition of endothelial cell injury. The protocol was the same as that described for Fig. 7. Data is depicted as the mean±SEM of the number of experiments indicated. The shaded area represents the mean±1 SEM of spontaneous release. The injury caused by neutrophils + LPS + FMLP was significant at P = 0.001(**). Significant inhibition was seen with the combination of AAPVCK + catalase + SOD (P = 0.05)(*) as well as AAPVCK alone (P = 0.05)(*). Neither TLCK alone nor the combination of TLCK + catalase + SOD inhibited injury to a statistically significant degree. Percent inhibition was calculated by averaging the mean percent inhibition from each experiment.

Since blood, which contains several inhibitors of elastase action, continually bathes endothelial cells, serum, plasma, and α_1 PI were also included in the assay to assess injury in the presence of physiologically relevant inhibitors. Autologous serum, made by adding calcium to platelet-rich, citrated plasma, was added with neutrophils and LPS to constitute 50% of the assay medium. Platelet-poor plasma was made from the same preparation by centrifugation of platelet-rich plasma. As shown in Table I, injury was not affected by the presence of serum, and the injury may even be slightly increased in the presence of plasma. The serine protease inhibitor α_1 PI, which inhibits trypsin, elastase, and tissue enzymes (40), also did not inhibit injury at concentrations similar to those measured in normal human plasma (1.5 mg/ml) (41).

Discussion

In this study we have employed an assay that measures injury to cultured human microvascular endothelial cells. This injury appears to represent cell lysis, since large amounts of a label that binds tightly to endothelial cells were found in the supernatant, and release of this label correlated well with release of the intracellular enzyme LDH. In addition, the pellet obtained after centrifugation of washes contained cell debris and not intact endothelial cells. The results of these experiments show that LPS greatly enhanced the endothelial injury caused by FMLP or C5a-stimulated neutrophils. The effects of the two stimuli were not merely additive, since neutrophils incubated with either LPS or FMLP alone caused minimal injury even in high concentrations.

Table I. Effect of Serum, Plasma, and α_1 PI on Endothelial Cell Injury

	% Buffer control	
Additions	N + LPS + FMLP	N + LPS + FMLP + addition
50% Serum (n = 3)	168.1±18.1	170.1±17.7
50% plasma (n = 4)	165.9±13.1	232.4±48.4
1.5 mg/ml α_1 PI (n = 3)	168.3±8.5	186.9±11.4

The protocol was the same as that described for Fig. 7. Data is depicted as the percentage of ^{111}In release by buffer control and is the mean±SEM of the number of experiments indicated. The percent ^{111}In release from endothelial cells by buffer controls for the serum experiments was 11.0±2.2%, for plasma 16.1±7.2%, and for α_1 PI 9.5±2.3%.

Instead, the sequential incubation of LPS followed by FMLP or C5a with neutrophils and endothelial cells appeared to evoke a synergistic response. The enhancement provided by LPS was evident at very low LPS concentrations (1–10 ng/ml) comparable to those described in human endotoxemic states (34). Similarly, neutrophil-mediated injury in the presence of LPS was evoked with as little as 10^{-8} M FMLP.

Our data, particularly the pretreatment studies presented here, imply that LPS enhanced injury through its effect on the neutrophil and not the endothelial cell, since neutrophils pretreated with LPS elicit the same magnitude of injury as the coincubation of neutrophils, endothelial cells, and LPS. However, we cannot completely rule out an effect of LPS directly on endothelial cells. Brigham and Meyrick (42) have suggested that granulocyte-mediated injury may not occur unless the endothelial cells have already been subtly harmed or altered. Thus, LPS may affect the endothelial cell so as to make it more susceptible to injury. In our system, LPS alone had no measurable injurious effect over a 4-h incubation (see Fig. 1) or, in unpublished studies, over a 24-h incubation (data not shown). Recent evidence suggests that, unlike bovine endothelial cells, human umbilical vein endothelial cells are not susceptible to injury by incubation with up to 100 μg/ml of LPS for 24 h (43). Our data would suggest that resistance to direct LPS toxicity is a feature of human microvascular cells as well. Although enhanced endothelial susceptibility in terms of a subtle alteration cannot be ruled out by either the apparent resistance to LPS alone or the pretreatment data, we suggest that, in this system, LPS acts on the neutrophil to enhance endothelial injury.

A number of groups have reported that neutrophils stimulated by a single agent are capable of injuring endothelial cells (18, 20). One factor that may contribute to these apparently discrepant results is that neutrophil isolation procedures have varied in different studies. We have shown (14) that neutrophils, prepared by a traditional method using Ficoll-Hypaque gradients, appeared to have been "primed" when subsequently tested for various in vitro functions, as compared with cells prepared by using plasma-Percoll gradients. One possible cause was exposure of neutrophils to trace concentrations of LPS, which were detected in reagents used in the traditional isolation method. These observations and the current investigation indicate that very different results may be obtained if the neutrophils have been primed during the isolation procedure. In particular, it may help explain why some workers have induced endothelial injury with a single stimulus, such as FMLP, C5a, or phorbyl myristate acetate (PMA). In addition, our studies here suggest that human umbilical vein endothelial cells, used commonly in studies of injury, were able to be injured by neutrophils stimulated only with FMLP.

This study further suggests that neutrophil-derived elastase is the primary agent of endothelial cell injury in this system. This conclusion is based on several pieces of evidence. The specific elastase inhibitor, AAPVCK, inhibited 60–70% of the neutrophil-mediated injury, whereas scavengers of oxygen metabolites had no effect on the injury. Neutrophils from a patient with chronic granulomatous disease produced injury similar to that produced by normal control neutrophils, and this injury was inhibitable by AAPVCK. Purified human neutrophil elastase caused damage in a neutrophil-free assay with a time course similar to that of stimulated neutrophils, and quite different from that of H_2O_2-induced injury. Further, AAPVCK inhibited elastase-induced injury to the same degree as it inhibited neutrophil-mediated injury.

Some investigators have reported that oxygen species, either H_2O_2 or O_2^-, were the causative agent of neutrophil-mediated injury to endothelial cells. Sacks and colleagues (18), using neutrophils stimulated with zymosan-activated plasma (18), and Yamada and his co-workers (44), employing neutrophils incubated with serum and large doses of LPS (44), have reported that neutrophils injure endothelial cells by a catalase- and SOD-inhibitable mechanism. Important differences in the assays may have some bearing on this apparent discrepancy in results. One factor is the type of endothelial cell chosen. Our assay employed human microvascular endothelial cells for the major portion of the work rather than the large vessel cells (either human umbilical vein or bovine pulmonary artery) that have been used by other investigators. In preliminary studies, reported here, umbilical vein endothelial cells were 10 times more sensitive to H_2O_2-induced injury than human microvascular endothelial cells. A mechanism for this observation was provided by a recent study that suggested that umbilical vein endothelial cells lack catalase (45). It is plausible, therefore, that the type of endothelial cell may, in part, determine the mechanisms by which they are injured. Although adipose tissue, from which the microvascular endothelial cells were isolated, is not commonly thought to be injured during endotoxemia, the inflammatory responses in which neutrophil-endothelial interactions are important occur largely within microcirculatory beds. Accordingly, the study of injury to microvascular endothelial cells may be particularly relevant to the understanding of in vivo vascular injury.

In addition to the type of endothelial cell studied, the stimulus used to activate the neutrophils may have an effect on the endothelial injury produced. Both Weiss et al. (46) and Martin (47) demonstrated catalase-inhibitable endothelial injury using PMA as a neutrophil stimulus. The time course of measurable injury was markedly different from that of the neutrophil-mediated injury that was detectable within 1 h after FMLP stimulation in our study. Weiss (46) reported measurable injury 3–4 h after addition of stimulus, and Martin (47) did not see any injury until after 8 h of incubation. It is known that PMA, though a good stimulator of oxygen metabolites, is a weak stimulus for azurophil granule release (48). Clearly, oxygen metabolites such as H_2O_2 can injure endothelial cells (see Fig. 6), and a massive stimulus to their production would be expected to induce cellular damage. Thus, injury stimulated by PMA may be more susceptible to inhibition by serum and oxygen radical scavengers, than that stimulated by FMLP or C5a as examined in this study.

Neutrophil proteases, particularly neutrophil elastase, have been implicated in neutrophil-mediated endothelial injury by Harlan and colleagues (20). In contrast to our results, however, these investigators found endothelial cell detachment to be the predominant response. Further, a recent study by these authors suggested that neutrophils (isolated by the Ficoll-Hypaque method) stimulated by FMLP may alter endothelial cells by oxygen radical-independent mechanisms (49).

These data provide support for the concept that neutrophil-mediated injury to endothelial cells may occur by many mechanisms, including both oxygen radical-dependent and -independent ones. We have previously reported (13) that LPS preincubation did not produce an increase in FMLP binding sites, but did increase the V_{max} of the NADPH oxidase. More recently, we have shown that LPS preincubation alters the neutrophil so

as to enhance subsequently evoked neutrophil elastase release (50). Similarly, LPS preincubation may increase neutrophil elastase content (51). Thus, several of the major injury-inducing substances of the neutrophil—oxygen metabolites and elastase—can be shown to be increased under conditions of LPS pretreatment. Nonetheless, other neutrophil products, such as neutrophil cationic proteins (see below) may be important. Further, the potential interactions between the different neutrophil products in terms of enhancing injury have not been well studied.

Even though, in our system, elastase can be implicated as the major injurious agent from neutrophils, it is not clear how elastase might produce endothelial injury. Bovine pulmonary artery endothelial cells have been shown to produce elastin (52). Although the elastin content of microvascular endothelial cells is unknown, since elastase is active on a wide range of substrates, the degradation of other strategically located cell surface proteins might produce injury. Elastase may also affect endothelial cells via other mechanisms. For example, LeRoy et al. (53) have recently suggested that the highly cationic nature of elastase may alter endothelial cell function. This report is interesting in light of the fact that in our studies, the specific elastase inhibitor AAPVCK was not able to completely inhibit injury mediated by either neutrophils or purified elastase. A potential complicating factor is the contamination of our elastase preparation by small amounts of LPS. Although we have been unable to detect direct toxic effects of LPS on human microvascular endothelial cells, we were concerned that the combination of LPS and elastase might alter the effect of elastase. To address the difference between elastase and H_2O_2, we have performed three preliminary experiments in which pretreatment of endothelial cells with LPS (10–1,000 ng/ml) for 1 h did not alter the subsequent response to elastase or H_2O_2. These data (combined with the pretreatment data reported herein) suggest that LPS effects on the endothelial cell are of little importance over the short time courses reported here, but do not address the potential contribution of longer LPS incubations.

Although we identified enzymatically active neutrophil elastase as the most important injury-causing constituent of the neutrophil in this system, there may well be other neutrophil products that are important in endothelial cell injury. In this regard, it is of interest that neither serum or plasma, which contain significant amounts of elastase inhibitor, nor relevant concentrations of purified α_1 PI, inhibited neutrophil-mediated injury. The reason for failure of the circulating antiproteases to inhibit are unclear. Recent reports have suggested that α_1 PI can be inactivated by neutrophil-derived oxygen metabolites (54, 55) or leukocyte-mediated proteinases (56). Thus, the neutrophil itself might inactivate functional α_1 PI. Additionally, close contact between neutrophil and endothelial cell may be required for injury. Inhibitors the size of α_1 PI may thus not have access to the interface where elastase action is critical (57).

Circulating endotoxin has been implicated in a variety of syndromes of man and animals in which the primary manifestation is vascular injury. These include ARDS (3) and both the general (7) and local Schwartzman reactions. In the aforementioned settings, the neutrophil has been felt to play an important role in the genesis of vascular injury, based on both morphologic criteria and depletion studies. However, as indicated, the mechanisms by which LPS promotes neutrophil-mediated vascular injury, and the mechanisms by which neutrophils injure vascular endothelium, remain unclear.

Recent work has focused on the ability of intravascular LPS to activate complement systemically. Several lines of evidence, however, suggest that complement activation alone may be insufficient to produce lung injury in either humans or animals. Animal studies from our laboratory (11) (submitted for publication) have shown that intravascular complement activation or infusion of purified fragments of C5 in rabbits fail to produce functional or morphologic evidence of lung injury, although neutrophils are effectively sequestered within the lung vasculature. It should be pointed out, however, that studies in rats have shown evidence of lung injury after intravascular complement activation (16). Studies in humans further suggest that the generation of C5a is, by itself, insufficient to produce ARDS (58). Similarly, systemic activation of complement in patients with nephritis due to nephritic factor (59) has not been shown to lead to lung injury. Taken together, these data imply the involvement of multiple factors, including the generation of complement-derived chemotactic factors, in the pathogenesis of ARDS.

In this setting, the ability of LPS to alter directly the behavior of neutrophils may be of considerable importance. We have presented data from rabbits suggesting that the intravascular infusion of LPS and complement-derived chemotactic factors produces neutrophil-dependent lung vascular permeability and endothelial injury (submitted for publication). In the present study, we suggest that only when neutrophils are incubated with endothelial cells in the presence of LPS can they be stimulated by chemotactic factors such as FMLP or C5a to injure endothelial cells. Further, this injury still occurred in the presence of 50% serum or plasma and a physiologically relevant concentration of α_1 PI, the most potent circulating elastase inhibitor. Accordingly, we suggest that LPS and chemotactic factors (or other neutrophil stimuli) may act together to produce neutrophil-mediated vascular injury, both in vivo and in vitro, and that this synergistic interaction may help explain the pathogenesis of vascular injury in endotoxemia in both man and animals.

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