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Phospholipase C and Perfringolysin O from *Clostridium perfringens* Upregulate Endothelial Cell-Leukocyte Adherence Molecule 1 and Intercellular Leukocyte Adherence Molecule 1 Expression and Induce Interleukin-8 Synthesis in Cultured Human Umbilical Vein Endothelial Cells

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Clostridium perfringens **phospholipase C (PLC) and perfringolysin O (PFO) differentially induced human umbilical vein endothelial cell expression and synthesis of endothelial cell-leukocyte adherence molecule-1 (ELAM-1), intercellular leukocyte adherence molecule-1 (ICAM-1), and interleukin-8 (IL-8). PLC strongly induced expression of ELAM-1, ICAM-1, and IL-8, while PFO stimulated early ICAM-1 expression but did not promote ELAM-1 expression or IL-8 synthesis. PLC caused human umbilical vein endothelial cells to assume a fibroblastoid morphology, whereas PFO, in high concentrations or after prolonged low-dose toxin exposure, caused cell death. The toxin-induced expression of proadhesive and activational proteins and direct cytopathic effects may contribute to the leukostasis, vascular compromise, and capillary leak characteristics of *C. perfringens* gas gangrene.**

World War I surgeons stationed at the allied casualty clearing stations recognized that wounds which severed main blood vessels were associated with massive or group gangrene, meaning that bacterial proliferation and muscle destruction easily and rapidly ensued throughout all muscle groups which had been cut off from the blood supply (10).

It was the careful observations of McNee and Dunn which provided insight into the method of the spread of gas gangrene into living, healthy tissue. Their 1917 report in the *British Medical Journal* described the histopathology of serial sections of single muscle bundles from freshly amputated limbs of soldiers with gas gangrene (10). They noted that, at the advancing edge of the infection, few organisms were present, that the tissue fibers appeared ischemic, and that a toxic fluid, formed in the gangrenous tissue behind the advancing edge of infection, spread between the fibers, breaking down the tissue and providing an optimal environment for the organisms to proliferate. They also noted that in these areas "leucocytes are generally conspicuous by their absence" from involved tissues but are seen in the interfascial planes (10). Subsequent experimental histopathology studies demonstrated that these pathologies could be reproduced by the injection of either crude (14) or recombinant clostridial phospholipase C (PLC; alpha toxin) (5) or perfringolysin O (PFO; theta toxin) preparations (6) into healthy muscle. In these later studies, leukocytes were commonly seen amassed within small vessels adjacent to the sites of toxin injection (5, 6, 14).

In the acute stage of inflammation, the vascular endothelium regulates the accumulation, margination, diapedesis, and chemotaxis of leukocytes into tissue by modulating the expression of surface molecules (e.g., selectins and integrins) and soluble factors (e.g., cytokines) (reviewed in reference 2). We hypothesized that toxin-induced dysregulation of these events could contribute to the observed vascular leukostasis and the absence of a tissue inflammatory response. In addition, progressive endothelial cell dysfunction could contribute to the rapidly advancing margins of the tissue necrosis characteristic of clostridial gas gangrene.

We investigated the effects of PFO and PLC on endothelial cell expression of endothelial cell-leukocyte adherence molecule 1 (ELAM-1) and intercellular adherence molecule 1 (ICAM-1) and on the synthesis of endothelial cell-derived interleukin-8 (IL-8).

(Portions of this work were presented in abstract form to the First International Conference on Molecular Biology and Pathogenesis of Clostridia, Rio Rico, Arizona, in January 1995 [5]).

Exotoxins. Recombinant PFO was provided by Rodney Tweten, University of Oklahoma (21, 22). The activity of stock PFO was determined by hemolysis of a 1% suspension of sheep erythrocytes and was found to be 12,800 hemolytic units (HU)/ml (specific activity, 4.3 HU/μg of protein). Recombinant PLC was provided by J. Tso, Protein Design Labs, Palo Alto, Calif. (20). The phospholipase activity of stock PLC, determined by a synthetic chromogenic substrate assay, was 284 U/ml (specific activity, 0.284 U/μg of protein). These assays have been previously described in detail (17). Both toxin preparations yielded a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining. PFO and PLC stocks were also assayed for the presence of endotoxin by the *Limulus* amoebocyte assay (Associates of Cape Cod, Woods Hole, Mass.) and found to contain 0.001 ng/HU and 22.7 ng/U of PLC, respectively.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were purchased as primary or secondary cultures from Clonetics, Inc., San Diego, Calif., and were cultured at 37°C in complete endothelial growth medium (EGM; Clonetics) in a standard, humidity-controlled tissue culture incubator. Cells of passage 2-5 were seeded into 96-well Primaria tissue culture

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plates (Falcon) and grown to confluence. Three to five days postconfluence, the monolayers were washed with prewarmed EGM and inspected for signs of contamination or loss of monolayer integrity. Irregular wells were not used.

Toxin-induced expression of ELAM-1 and ICAM-1. The cell-based enzyme-linked immunosorbent assay (ELISA) system employed in this study was a modification of the methods of Colden-Stanfield et al. (8). Briefly, 20 μl of 10× agonist was added to 180 μl of EGM in the first column of wells, mixed, and serially diluted twofold across the plate. Wells containing blank EGM (negative control) or 100 U of tumor necrosis factor alpha (TNF-α; Cetus Corp., Emeryville, Calif.; 41.7 pg/U as a positive control) per ml were included on each plate. Additionally, to ensure that the observed effects were not due to the presence of contaminating lipopolysaccharide (LPS), the ELISA for ELAM-1 expression was performed with 0.025 U/ml of PLC, heat-inactivated PLC, or LPS (*Escherichia coli* 0113:H10; 0.2 to 100 μg/ml; Associates of Cape Cod). After 4 h (or other lengths of time as indicated in Fig. 2) at 37°C in 5% CO_2, the monolayers were visually inspected for cell loss or other cytopathic effects and culture supernatants from intact wells were removed and frozen at −70°C for IL-8 determinations. The monolayers were washed three times with Dulbecco's phosphate-buffered saline (DPBS) containing 1% (wt/vol) bovine serum albumin (DPBS-BSA). All residual fluid was carefully aspirated from each well with a Pasteur pipette after the last wash. The following steps were carried out at room temperature with three washes of DPBS-BSA between steps. Monolayers were fixed for 15 to 20 min with 2% (wt/vol) paraformaldehyde. Wells were then filled with DPBS-BSA for 30 min to block nonspecific binding. The primary antibodies, anti-ELAM-1 (clone 1.2B6, 0.25 μg/50 μl; Harlan Bioproducts/Serotec, Indianapolis, Ind.) or anti-ICAM-1 (clone B-C14, 0.5 μg/50 μl; Harlan) were added to the upper and lower halves of each plate, respectively, for 1 h. One hundred microliters per well of peroxidase-conjugated goat anti-mouse immunoglobulin G (Zymed Corp, South San Francisco, Calif.) diluted 1:1,000 in DPBS-BSA was added, and plates were incubated for 1 h. Plates were washed three times with DPBS without BSA, and 2,2′-azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS; 0.5 mg/ml; Zymed) in 0.1 M citrate buffer (pH 4.0) containing 0.03% hydrogen peroxide substrate was added. Color was allowed to develop for 10 to 20 min and the A_{405} was read on an ELISA plate reader.

This assay provides relative values for changes in adherence molecule expression. Baseline absorbance values reflect differences in the constitutive expressions of ELAM-1 and ICAM-1 on unstimulated endothelial cells (2).

Toxin-induced IL-8 synthesis. Replicate supernatant fluids from stimulated HUVEC were pooled, centrifuged to remove particulates, and frozen at −70°C. IL-8 levels were determined in duplicate for each sample by commercial ELISA (R&D Systems, Minneapolis, Minn.).

Three experiments were performed using HUVEC from three different donors. ELAM-1 and ICAM-1 upregulation was determined in triplicate for each experiment, and the values reported are the means of these determinations ($n = 9$) ± the standard error of the mean (SEM). IL-8 levels (in picograms per milliliter) are expressed as the mean of duplicate samples of pooled replicate supernatants ± SEM. Statistical significance was determined by one-way analysis of variance with Dunnett's test with the level of significance chosen as $P < 0.05$.

The exposure of HUVEC to low concentrations of PLC (≤0.02 U/ml) for 4 h increased ELAM-1 expression by three- to fourfold ($P < 0.05$) (Fig. 1). ELAM-1 expression induced by 0.025 U/ml of PLC peaked at 4 h and gradually returned to baseline by 24 h (Fig. 2A). Maximal PLC-induced ICAM-1 expression occurred at 8 to 12 h and remained elevated for 24 h (Fig. 2A) ($P < 0.05$). PFO (0.25 HU/ml) elicited a small but significant increase in ICAM-1 expression after 4 h (Fig. 1) ($P < 0.05$) which was maximal at 12 h (Fig. 2B). PFO did not induce ELAM-1 expression at the times or concentrations tested (Fig. 1 and 2B).

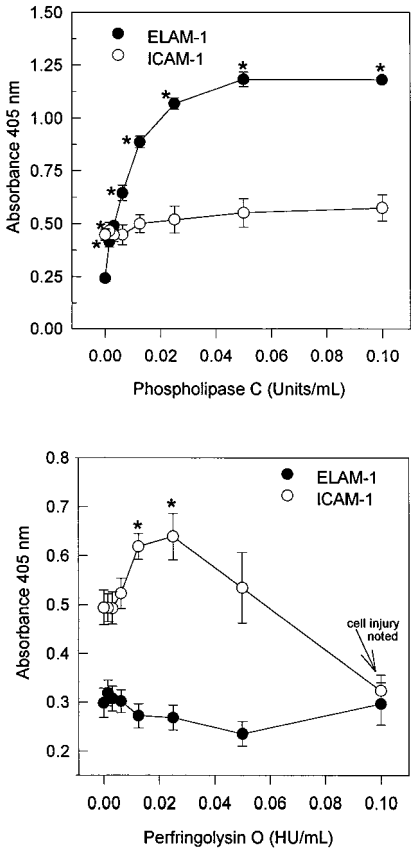

FIG. 1. Dose responses of *C. perfringens* PLC- and PFO-induced expression of ELAM-1 and ICAM-1 on HUVEC. Confluent monolayers of HUVEC grown in 96-well plates were exposed for 4 h to various concentrations of either PLC or PFO. The expression of ELAM-1 and ICAM-1 was measured by ELISA with primary antibodies specific for these adherence molecules. Experiments were performed in triplicate using HUVEC from three different donors. Values expressed are the means of these determinations ($n = 9$) ± SEM. *, $P < 0.05$, compared with the value for unstimulated control. PFO at 0.1 HU/ml for the 4-h incubation period was directly cytotoxic to HUVEC, causing a loss of approximately 50% of cells.

PLC at doses greater than 0.00625 U/ml for 24 h did not result in discernable cell loss; however, HUVEC reorganized from their normal cobblestone shape into a spindle-shaped, fibroblastoid morphology (not shown), suggesting that PLC may affect the actin filaments. High concentrations of PFO (>0.1 HU/ml) for 4 h caused HUVEC death and detachment. Similarly, prolonged incubation (>12 h) of HUVEC with low-dose PFO caused marked cellular destruction, making determinations of adherence molecule expression at 24 h impossible.

PLC (0.025 U/ml) elicited a strong ELAM-1 response ($P < 0.001$ versus the unstimulated control) which was comparable to that produced by optimal doses of two known activators of endothelial cell adherence molecule expression, i.e., TNF and LPS (Fig. 3). This response was specific to PLC activity and not

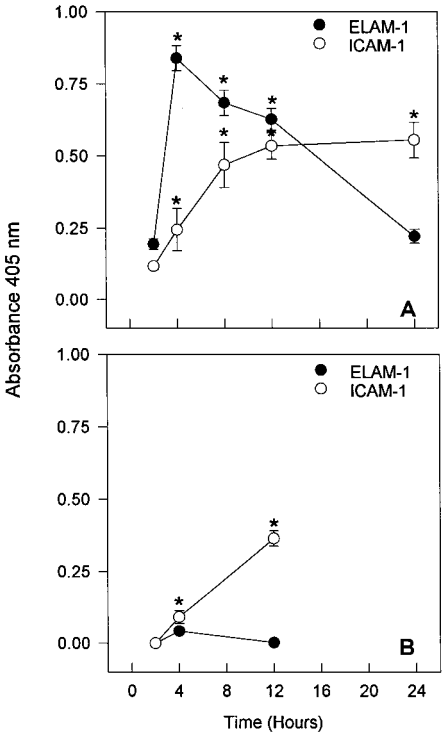

FIG. 2. Time courses of *C. perfringens* PLC- and PFO-induced expression of ELAM-1 and ICAM-1 on HUVEC. Confluent monolayers of HUVEC grown in 96-well plates were exposed to either (A) 0.025 U of PLC per ml or (B) 0.025 HU of PFO per ml for 2, 4, 8, 12, or 24 h. The expression of ELAM-1 and ICAM-1 was measured by ELISA with primary antibodies specific for these adherence molecules. Experiments were performed in triplicate using HUVEC from three different donors. Values expressed are the means of these determinations minus the mean unstimulated control value at each time point ± SEM. *, $P < 0.05$, compared with the value for the respective unstimulated control. Direct cytotoxicity of PFO at 24 h resulted in significant cell loss, making determinations at this time impossible.

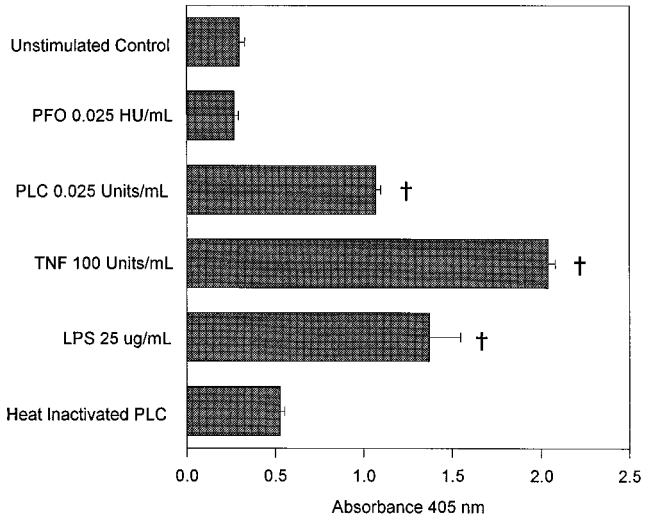

FIG. 3. Comparison of *C. perfringens* PLC and PFO with other known endothelial cell activators to stimulate ELAM-1. Confluent monolayers of HUVEC grown in 96-well plates were exposed for 4 h to either blank media (unstimulated control), PFO at 0.025 HU/ml, PLC at 0.025 U/ml, recombinant human TNF-α at 100 U/ml, *E. coli* LPS at 25 μg/ml, or heat-inactivated PLC. Surface ELAM-1 expression on HUVEC was determined by ELISA. Experiments were performed in triplicate using HUVEC from three different donors. Values expressed are the means of these determinations ± SEM. † = $P < 0.001$.

due to LPS contamination since (i) heat inactivation of PLC reduced the effect on ELAM-1 expression (Fig. 3) and (ii) the dose of PLC used contained 0.57 ng of LPS per ml, a concentration of LPS which was too low to induce ELAM-1 expression (data not shown).

PLC, but not PFO, induced IL-8 synthesis by HUVEC during the 4-h period of toxin stimulation (Fig. 4) ($P < 0.001$ versus the unstimulated control). As with ELAM-1 expression, the amount of IL-8 produced in response to PLC was comparable to that elicited by TNF and LPS and heating the PLC diminished this effect.

Gas gangrene due to *Clostridium perfringens* is an aggressive soft tissue infection associated with shock, rapidly developing ischemic tissue necrosis, and death in approximately 30% of patients (15). Among survivors, morbidity is extensive; radical amputation on an emergent basis remains the single best treatment since margins often advance several inches per hour despite appropriate antibiotic coverage (15). The histopathology of gas gangrene is unique and distinctly different from infections caused by bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, or *Streptococcus pneumoniae*. In these cases, a luxuriant pyogenic response occurs at the site of infection. In contrast, clostridial myonecrosis is remarkable for the absence of acute inflammatory cells (10, 15). Leukocytes, when present, are localized between fascial planes (10) and within small vessels near the demarcation between healthy and necrotic tissues (6).

We hypothesized that toxin-induced dysregulation of the normal, physiologic mechanisms of leukocyte accumulation, adherence, and extravasation, which orchestrate the pyogenic responses to other infections, could, in part, explain the leukostasis and antiinflammatory response characteristic of clostridial gas gangrene.

Successful transmigration of leukocytes through the vessel

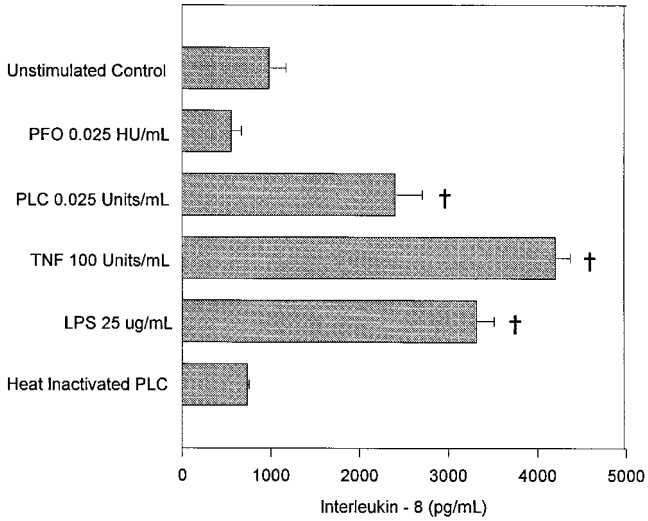

FIG. 4. Comparison of *C. perfringens* PLC and PFO with other known endothelial cell activators to stimulate endothelial cell synthesis of IL-8. Confluent monolayers of HUVEC grown in 96-well plates were exposed for 4 h to either blank media (unstimulated control), PFO at 0.025 HU/ml, PLC at 0.025 U/ml, recombinant human TNF-α at 100 U/ml, *E. coli* LPS at 25 μg/ml, or heat-inactivated PLC. Supernatant fluids from replicate wells were pooled, centrifuged to remove particulates, and frozen at −70°C. IL-8 was measured in duplicate by a commercial ELISA kit specific for this cytokine. Experiments were performed using HUVEC from three different donors. Values expressed are the means of these determinations ± SEM. † = $P < 0.001$.

and to the site of infection is the culmination of a complex cascade of both leukocyte- and endothelial cell-dependent adherence and activational events (reviewed in reference 2). Initially, the circulating, unactivated leukocyte is tethered to the activated endothelial cell and rolls along the vessel's luminal surface, processes mediated by selectins. Tethering results in juxtacrine activation of the leukocyte by platelet-activating factor, the functional upregulation of leukocyte CD11b/CD18, and firm adhesion to ICAM-1 constitutively expressed on the endothelial cell (12). The local production of cytokines (e.g., TNF and IL-1) augments the inflammatory response by stimulating endothelial cells to produce the neutrophil chemoattractant-activator, IL-8, to increase ICAM-1 expression, and to transiently express ELAM-1. Strongly adherent, activated leukocytes move to endothelial cell junctions and emigrate through the vessel, aided by platelet-endothelial cell adhesion molecule-1–CD31) (4, 11).

Previous studies in our laboratory investigated the effects of clostridial exotoxins on leukocyte-dependent mechanisms of neutrophil adherence to the endothelium (6, 7). In this study, we asked what effects PLC and PFO have on the expression of proadhesive proteins by the endothelial cell. PLC strongly induced the expression of ELAM-1 and ICAM-1 on HUVEC (Fig. 1). The magnitude of these responses was similar to that reported in other studies using either TNF, IL-1, or LPS from gram-negative organisms (3, 13).

PLC was also a potent inducer of endothelial cell-derived IL-8. As with ELAM-1 expression, the dynamics of PLC-induced IL-8 synthesis was comparable to that induced by TNF or LPS (Fig. 4 and reference 18). The local production of IL-8 in gas gangrene could amplify the recruitment of leukocytes and prime them for enhanced respiratory burst activity. These IL-8 effects, coupled with the toxin-induced hyperadhesion of leukocytes, enhanced respiratory burst activity (6, 16), and chemotaxis deficits (6, 16), could result in neutrophil-mediated vascular injury.

In contrast to PLC, PFO caused a modest but significant increase in ICAM-1, had no effect on ELAM-1 expression, and did not induce detectable IL-8 synthesis. These findings support the concept that endothelial cell responses are dictated by the nature of the inflammatory signal (2). However, the intramuscular injection of PFO into mice produces marked vascular leukostasis adjacent to the site of toxin injection (6), suggesting that, in vivo, other mechanisms may be operative. For instance, we have previously shown that PFO caused the functional upregulation of leukocyte CD11b/CD18 (6). In addition, PFO stimulated cultured endothelial cells to synthesize platelet-activating factor (23), a proadhesive lipid autocoid which contributes to early neutrophil adherence to the endothelium (24). Further, PFO has potent degranulating activity and could potentially stimulate endothelial cells to express P-selectin, a preformed adherence molecule stored within Weibel-Palade bodies (9).

Direct toxin-induced cytopathic effects on endothelial cells may also contribute to vascular abnormalities associated with gas gangrene. In this study, PLC at high concentrations caused endothelial cells to undergo profound shape changes similar to those described following prolonged TNF or gamma interferon exposure (19). In vivo, the conversion of endothelial cells to this fibroblastoid morphology could contribute to the localized vascular leakage and massive swelling observed clinically with this infection. Similarly, the direct cytotoxicity of PFO could disrupt endothelial integrity and contribute to progressive edema. These cytopathic effects could expose the subendothelium and initiate coagulation and platelet aggregation, thus compounding microvascular occlusion.

Thus, PLC and PFO may cause local, regional, and systemic vascular dysfunction. The local absorption of exotoxins within the capillary beds could affect the physiological function of the endothelium lining the postcapillary venules, resulting in the impairment of phagocyte delivery at the site of infection. Toxin-induced endothelial dysfunction and microvascular injury could also cause loss of albumin, electrolytes, and water into the interstitial space, resulting in marked localized edema. These events, combined with leukostasis within the venule, would increase venous pressures and favor further loss of fluid and protein in the distal capillary bed. Ultimately, a reduced arteriolar flow would impair oxygen delivery, thereby attenuating phagocyte oxidative killing and facilitating anaerobic glycolysis of muscle tissue. The resultant drop in tissue pH, together with reduced oxygen tension, might further decrease the redox potential of viable tissues to a point suitable for growth of this anaerobic bacillus. As infection progresses and additional toxin is absorbed, larger venous channels would become affected, causing regional vascular compromise, increased compartment pressures, and rapid anoxic necrosis of large muscle groups. When toxins reach arterial circulation, systemic shock and multiorgan failure rapidly ensue and death is common (1).

In summary, the results of this study suggest that exotoxins produced by *C. perfringens* have profound effects on endothelial cell-dependent mechanisms of leukocyte accumulation and adherence. The effects of PLC and PFO on numerous other proadhesive and transmigrational cell-cell interactions remain to be investigated. A more complete understanding of the pathophysiology of these vascular events may provide a rationale for antiadhesion therapy. Such novel treatment strategies may greatly reduce the necessity for amputating limbs and may improve patient survival.

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