mediated reduction of glutathione represents a target that is common to all trypanosomal and leishmanial diseases. In the absence of this essential cofactor, or if its reductase were inhibited, these parasites would be unable to maintain their intracellular pool of glutathione in the reduced form. On the basis of previous studies (*20, 21*), depletion of total GSH or alterations in the GSH:GSSG ratio within the cell would be expected to be deleterious to these organisms. Several potential sites for drug development can be identified, including inhibition of synthesis of trypanothione and inhibition of trypanothione reductase. The details of the pathway of synthesis of trypanothione remain to be elucidated, but initial studies with [^{3}H]spermidine have shown that this polyamine can be incorporated into trypanothione. Since α-difluoromethylornithione inhibits spermidine synthesis in trypanosomes (*22*), the selective toxicity of this drug, now undergoing clinical trials (*23*), could be due in part to inhibition of trypanothione synthesis.

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13. Cofactor was desalted as described above and concentrated by evaporation under reduced pressure. Approximately 20 μg was electrosprayed onto aluminized polyester film and mass spectra were obtained with the Rockefeller University ^{252}Cf fission fragment ionization time-of-flight mass spectrometer [B. T. Chait, B. F. Gisin, F. H. Field, *J. Am. Chem. Soc.* **104**, 5157 (1982)].
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Disorganization of Cultured Vascular Endothelial Cell Monolayers by Fibrinogen Fragment D

Abstract. *Fibrinogen fragment D, which is heterogeneous, has several important biological functions. Human fibrinogen fragments D_{94} (molecular weight, 94,000), D_{78} (78,000), and E (52,000) were purified. Fragments D_{78} and D_{94} but not purified fibrinogen or fragment E specifically caused disorganization of bovine aortic endothelial cells cultured as monolayers. Within 2 hours of exposure to pathophysiological concentrations of fragment D, the confluent endothelial cells retracted from each other and projected pseudopodia. These disturbed cells subsequently became rounded and detached from the substrate. The actin present in stress fibers in stationary monolayer cells was diffusely redistributed in cells with fragment D–induced alterations in morphology. This effect was not observed in monolayers of kidney epithelial cells. The results demonstrate a specific effect of fibrinogen fragment D on the disorganization of cultured vascular endothelial cell monolayers and suggest that fragment D plays a role in the pathogenesis of syndromes with vascular endothelial damage.*

Recent studies have demonstrated that fibrinogen fragment D is directly involved in several biological activities. Purified fibrinogen fragment D stimulates the biosynthesis of fibrinogen (*1, 2*) and the proliferation of human hemopoietic cells in vitro (*3*) and contains the peptide essential for binding this fragment to D_2 Newman staphylococci (*4*) and human platelets (*5*). The fragment also inhibits fibrin monomer polymerization (*6*). Intravenous infusion of purified fragment D monomer induces hypoxemia and tachypnea and increases capillary permeability in rabbits (*7, 8*). The plasma level of fragment D antigen is markedly increased in patients with adult respiratory distress syndrome (ARDS) (*9, 10*). High levels of this fragment have been observed in patients with thrombotic thrombocytopenic purpura (TTP) (*11*) and in patients with disseminated intravascular coagulopathy (DIC) (*12*). These syndromes share the salient features of elevated fibrinogen-fibrin degradation products, including fragment D, and severe endothelial cell abnormalities. The etiology of the syndromes is not known. Because of this association, we investigated the hypothesis that fibrinogen frag-

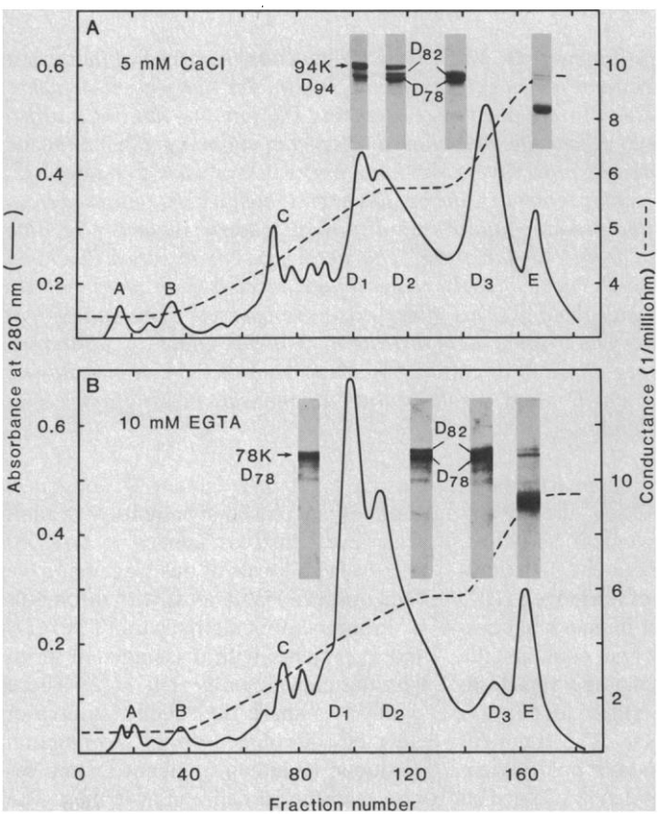
ment D directly disorganizes the architecture of the endothelial cell monolayer.

Fibrinogen degradation fragments were prepared by the method of Doolittle and *et al.* (*13*) with certain modifications (*1*). Human fibrinogen was purchased (Kabi) or purified from plasma (*14*). Fibronectin (molecular weight, 220K), removed by cold precipitation, was found to be <0.5 percent of total protein by reduced sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Thrombolysin (lot 0960W, Merck Sharp & Dohme)—which contains human plasminogen, plasmin, and streptokinase—or purified plasmin (Kabi) were used to digest the purified fibrinogen.

Purified human fibrinogen (200 mg) was suspended in 20 ml of buffer (0.15*M* NaCl and 0.05*M* tris-HCl, *p*H 7.2) in the presence of 0.005*M* $CaCl_2$ or 0.010*M* EGTA and digested with 0.003 casein unit of plasmin per milligram of fibrinogen at 37°C for 15 hours. The reaction was stopped by adding ε-aminocaproic acid to a final concentration of 0.2*M* and phenylmethylsulfonyl fluoride to a final concentration of 0.1 m*M*. The digest was chromatographed on a DEAE-cellulose DE52 column (2.5 by 45 cm) with the descending technique by using a linear double gradient of 0 to 0.09*M* NaCl and 0.09 to 0.17*M* NaCl in 0.01*M* $NaHCO_3$-Na_2CO_3 buffer (*p*H 8.9) (*13*). The protein was assayed by absorbance at 280 nm. Fraction peaks were assayed for identification by slab SDS-PAGE (*15*) and pooled. Pooled fractions in standard cellulose membranes were dialyzed extensively against 100 volumes of distilled water with four changes at 4°C over 24 hours, lyophilized, and stored at −20°C. Conductance was measured with a CDM 2d conductivity meter (Radiometer).

A 15-hour plasmic digestion of fibrinogen in the presence of 5 m*M* $CaCl_2$ was chromatographed on DEAE-cellulose columns with a linear ionic double gradient. This procedure yielded seven major peaks (Fig. 1A). Fragment D eluted in three peaks. The first of these was designated D_1; examination of the peak fractions by SDS-PAGE showed the major band to have a molecular weight of 94K (fragment D_{94}) (Fig. 1A). Peak D_1 was contaminated 5 to 10 percent by an adjacent peak, designated D_2. The second peak, D_2, contained three bands: D_{94}, D_{82} (82K), and D_{78} (78K). The third peak, designated D_3, contained two major bands, D_{82} and D_{78} (Fig. 1A). Reduced SDS-PAGE of fragments D_{94}, D_{82}, and D_{78} showed that the difference in the molecular weights resides in differences in the length of the γ chain (*16*). The last peak to elute, designated E, contained a single component with a molecular weight of 52K (Fig. 1). When identical plasmic degradation of fibrinogen was performed in a calcium-free environment with 10 m*M* EGTA, predominantly peak D_1 resulted, containing a single band of 78K (Fig. 1B), and it eluted first. In the presence of EGTA fragment D_{94} was absent. In the presence of EGTA fragment E (52K) was the last peak to elute (Fig. 1B), as was observed in the presence of 5 m*M* $CaCl_2$. These results confirm the observation (*17*) that calcium provides a protective effect, limiting degradation of fibrinogen by plasmin.

Bovine aortic endothelial cells from B. Glaser were cultured (*18*) on cover slips with and without a collagen coating at 37°C. Similar results were obtained with cells from passages 5 and 12. Endothelial cells were grown to confluence (*18*) and were incubated with purified human fibrinogen fragments E, D_{94}, and D_{78} in various concentrations. All components of the incubation mixture were negative for endotoxin by the *Limulus* assay (*1*, *2*). At least 10 percent of the endothelial cells showed retraction from each other within 2 hours of incubation with fragments D_{94} or D_{78} (200 μg/ml) in the presence or absence of the collagen substrate. After 15 hours of incubation at 37°C, endothelial cells exposed to frag-



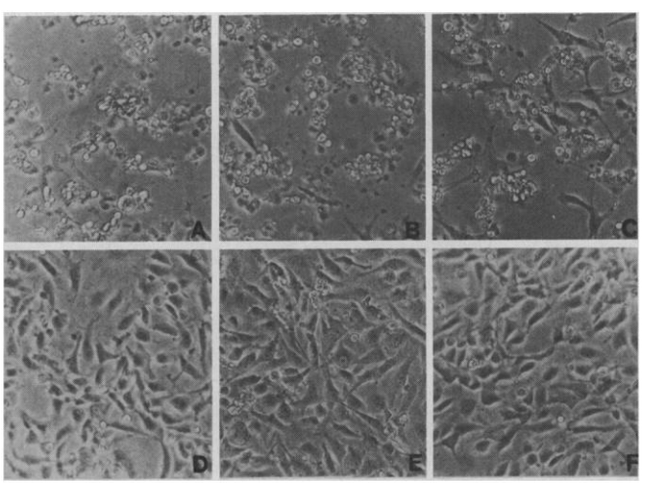


Fig. 1 (left). (A) Purification of fibrinogen fragments from fibrinogen plasmic digestion in the presence of 5 m*M* $CaCl_2$. Protein concentration was measured by absorbance at 280 nm and conductivity in reciprocal milliohms. Fibrinogen digest (200 mg) was chromatographed on a DEAE-cellulose DE52 column (2.5 by 45 cm). Each fraction contained 10 ml. Fragments D eluted in three peaks: D_1, D_2, and D_3. Peak D_1 contained fragment D_{94} and about 5 percent D_{82}. Peaks D_2 and D_3 contained fragment D_{78} but not D_{94}. Fragment E eluted in peak E (52K). (B) Purification of fibrinogen fragments from fibrinogen plasmic digestion in the presence of a calcium chelator, EGTA. Chromatography was performed as described above. Peak D_1 contained exclusively fragment D_{78}. Peaks D_2 and D_3 contained fragments D_{82} and D_{78}. Fragment E eluted in peak E (52K). Fig. 2 (right). Effect of fibrinogen and fibrinogen fragments on endothelial cell monolayers. Fibrinogen or fibrinogen fragments were added to the culture medium (*18*) of endothelial cell monolayers and incubated at 37°C for 15 hours. Fragment D_{94} (200 μg/ml) disrupted the monolayers (A and B), as did fragment D_{78} (200 μg/ml) (C); no effects were observed with fragment E (200 μg/ml) (D), fibrinogen (800 μg/ml) (E), or in the control (F) (magnification ×100).

ment D_{94} (Fig. 2, A and B) or D_{78} (Fig. 2C) showed extensive retraction and rounding, in contrast to the intact monolayers exposed to fragment E (200 μg/ml) (Fig. 2D) or fibrinogen (800 μg/ml) (Fig. 2E) or the control (Fig. 2F). The molar ratio of fragment E to D_{94} or D_{78} to fibrinogen in these experiments was approximately 2 to 1 to 1.

Figure 3 shows the specific disruptive effect of fragment D_{94} or D_{78} on endothelial cell monolayers. Denaturation by heating at 100°C for 20 minutes destroyed this effect. The specificity was further demonstrated by the lack of disruption of monolayers of kidney epithelial PtK2 (*15*) cells by fragment D_{94}. The possibility that contaminated plasmin caused the retraction of cells was excluded by the observation that the purified fragments did not have plasmin activity, as determined by hydrolysis of chromogenic substrate S2251 (Kabi). Thrombolysin and purified plasmin (100 μg/ml) did not have any disruptive effect on the monolayers (*19*). Cells that finally became rounded and that detached from the substrate were 90 percent replatable and excluded Trypan blue, suggesting that the effect of fragments D_{94} or D_{78} at the concentrations examined was not cytotoxic and was reversible. Preliminary studies show that purified fibrin D dimer (*2*) also causes disruption of endothelial cell monolayers, indicating that the cross-link site on the γ chain is not important for the disruption. Further evidence that cross-linking of the γ chain is not important is provided by the effectiveness of fragment D_{78}, which does not contain the cross-link site (*20*), in disrupting the monolayers.

A dose-response effect of fragment D_{94} on the disruption of the endothelial cell monolayer was demonstrated (Fig. 3). The concentration of fragment D causing altered morphology (Fig. 4) in 50 percent of cells exposed was 80 μg/ml. Patients afflicted with ARDS show an average concentration of fragment D antigen of 40 μg/ml 36 hours after onset of the syndrome (*9*). In vivo, fragment D antigen may be a mixture of D monomer and D dimer. The effect of D dimer on endothelial cells is not known. Direct interaction of fragment D with endothelial cells resulting in disruption of monolayers suggests that direct interaction of fragment D with endothelial cells plays a role in the pathogenesis of ARDS and perhaps DIC and TTP.

Nitrobenzoxadiazole (NBD)-phallacidin (Molecular Probes) staining of actin was also performed (*21*). Endothelial cells grown on cover slips were fixed in acetone (−20°C) for 2 minutes and rehydrated in 10 m*M* sodium phosphate and 150 m*M* NaCl (*p*H 7.4). Cover slips were stained with NBD-phallacidin at room temperature for 20 minutes, washed, and wet-mounted for fluorescence microscopy and photography (*15, 22*).

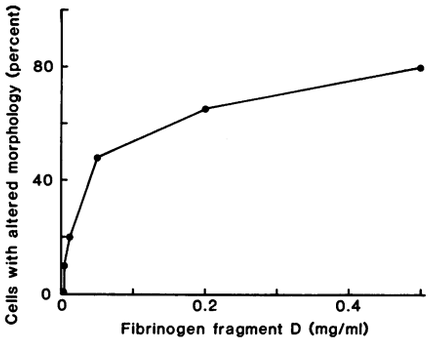


Fig. 3. Dose-response effect of fragment D_{94} disruption of endothelial cell monolayers. The ordinate shows the percentage of rounded cells in a total of 500 cells at different concentrations of fragment D_{94}.

Cells not exposed to fragment D_{94} or D_{78} remained flattened and retained a normal distribution of actin stress fibers (Fig. 4A). Within 2 hours of incubation at 37°C with fragment D_{94} or D_{78}, cells retracted from surrounding cells and projected pseudopodia; these changes were associated with diffuse cortical distribution of F-actin (Fig. 4B). Further retraction (Fig. 4C) was characterized by extensive surface blebbing and diffuse distribution of F-actin. The cells that became rounded and that subsequently detached from the substrate displayed diffuse F-actin staining with some punctate fluorescence (Fig. 4D). Clearly, stress fibers were lost when retraction, projection of pseudopodia, and rounding of endothelial cells took place.

Actin stress fibers are essential in maintaining the structural integrity of endothelial cells in vivo and in vitro (*23*). The distribution of these fibers was correlated with cell motility (*22*); endothelial cells observed to retract on exposure to fragment D displayed diffuse F-actin. Motility involved in retraction may be reflected in the redistribution of F-actin from stress fibers to cortical areas. The molecular mechanism by which fragment D alters actin distribution remains to be established.

In a study by Kadish *et al.* (*24*), fibrin clots rapidly disorganized monolayers of cultured bovine aortic endothelial cells but did not affect the architecture of eight other cell types. Fibrin clots caused retraction of endothelial cells and induced increased random motility of cells. The active principle from the fibrin clot was not identified, but it is likely to be a plasminolytic fibrin product since intravascular fibrin deposition and fibrinolysis are required for mediation of lung vascular injury (*25*). Busch and Gerdin (*26*) showed that fibrinogen degradation products of low molecular weight cause retraction of human endothelial cells. These small dialyzable peptides are not well defined and are not responsible for the effects we observed, since our purified fibrinogen fragments were extensively dialyzed. The purified fragments did not show small peptides on SDS-PAGE at the dye front or on staining nitrocellulose membrane blots of the fragments with rabbit antibody to human fibrinogen and ^{125}I-labeled protein A. The submolecular site of origin of the

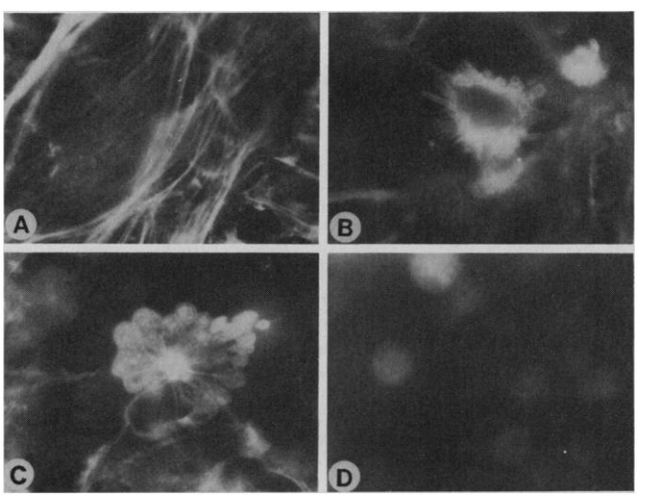


Fig. 4. Effect of fragment D_{94} on F-actin distribution in cultured endothelial cells. (A) Control endothelial cells not exposed to fragment D_{94} show typical actin stress fibers stained with NBD-phallacidin. (B) Cells exposed to fragment D_{94} (200 μg/ml) retract, project pseudopodia, and demonstrate diffuse cortical distribution of F-actin 2 hours after incubation. (C) Blebbing of the cell surface is noted with retraction. (D) With 15 hours of exposure to fragment D_{94} the cells become rounded and show diffuse and some punctate F-actin staining.

peptides of Busch and Gerdin (*26*) was not specified, but they may be polypeptides from the biologically active sites on fragment D.

Our results demonstrate that fibrinogen fragment D is an active principle in the specific disorganization of cultured vascular endothelial cells and suggest that fragment D plays a role in the pathogenesis of syndromes with vascular endothelial damage. Further investigations of fragment D with human capillary endothelial cells are necessary, as the pathological effects of fragment D may vary with the types and species of origin of the endothelial cells. The significance of fragment D in vivo and of its potential complex with fibrin monomer and fibrin fragment D dimer in the pathogenesis of vascular endothelial damage remain to be established.

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Protection from Genital Herpes Simplex Virus Type 2 Infection by Vaccination with Cloned Type 1 Glycoprotein D

Abstract. *Guinea pigs were vaccinated with truncated herpes simplex virus type-1 (HSV-1) glycoprotein D produced in the genetically engineered mammalian cell line gD10.2. Vaccinated animals formed antibodies that neutralized both HSV-1 and herpes simplex virus type 2 (HSV-2) in an in vitro neutralization assay. Vaccinated animals were challenged with HSV-2 by intravaginal infection. Animals that received the immunogen in Freund's complete adjuvant were completely protected from the clinical manifestations of genital HSV-2 infection. Animals that received the immunogen incorporated in alum adjuvants were partly protected from clinical disease; the infections that did develop were significantly less severe than those that occurred in control animals injected with adjuvant alone. The results demonstrate that immunization with a purified viral protein can provide significant protection against primary genital infection by HSV-2 in guinea pigs.*

The incidence of genital infections resulting from herpes simplex viruses (HSV's) has increased considerably over the past 20 years (*1*). Because HSV DNA is oncogenic in vitro (*2*), traditional methods of vaccine production must be approached with caution. Thus live attenuated virus vaccines, killed virus vaccines, and subunit vaccines consisting of viral proteins isolated from virus-infected cells are unlikely to gain acceptance until it can be shown that such preparations are free of potentially transforming fragments of viral DNA. Recent advances in recombinant DNA technology now make it possible to produce virtually unlimited quantities of purified viral antigens without resorting to large-scale cultivation of the infectious pathogen. Using this technology, we have attempted to develop an effective subunit vaccine to provide protection from primary infection by HSV type 1 (HSV-1) and HSV type 2 (HSV-2).

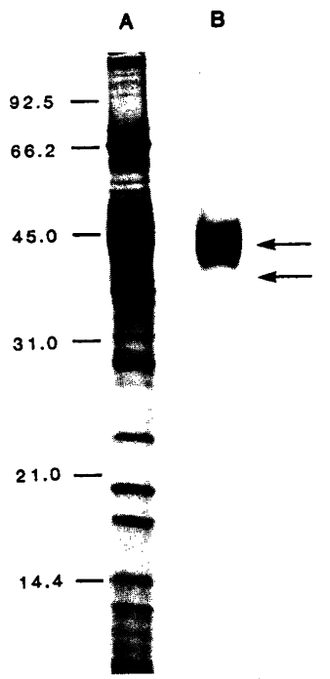

Fig. 1. Purification of truncated HSV-1 gD produced by the gD10.2 cell line. Serum-free culture medium conditioned by growth of the gD10.2 cell line was harvested and concentrated (*9*) and was further purified by affinity chromatography (*11*). Samples of the crude starting material and the purified product were analyzed on 10 percent polyacrylamide gels (*15*) and silver-stained. (A) Unfractionated culture medium from the gD10.2 cell line. (B) Purified gD-1 eluted from the affinity column. Arrows indicate the mature, fully glycosylated gD-1t (upper arrow) and the incompletely glycosylated gD-1t precursor (lower arrow). Mobilities (K) of molecular size markers are indicated in the left margin.

Both HSV-1 and HSV-2 are large enveloped DNA viruses that contain five or six major envelope glycoproteins, designated gA/B, gC, gD, gE, gF, and gG (*3*). In previous studies we (*4*) and others (*5*) cloned and determined the complete nucleotide sequence of the genes encoding glycoprotein D (gD) of HSV-1 and HSV-2. The sequence data confirmed immunologic data indicating that the two proteins were closely related (*6–7*) and suggested that antibodies elicited by either protein should cross-protect against HSV-1 and HSV-2 infections. In subsequent studies we demonstrated that both a full-length (*8*) and a truncated (*9*) gD-1 gene could be transferred into Chinese hamster ovary (CHO) cells and that permanent cell lines that constitutively synthesized a full-length, membrane-bound form of gD-1 and a truncated, secreted form of gD-1 (gD-1t) could be constructed. In addition, we found that vaccination of mice with gD-1t elicited an effective cross-neutralizing immune response that provided complete protection against a lethal intraperitoneal infection by HSV-1 or HSV-2.

Although the mouse provided a convenient model system in which to screen our early vaccine preparations, the guinea pig model more closely resembles human infections in that intravaginal infection of these animals with HSV-2 (*10*) leads to the development of acute vesi-