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THROMBIN PRODUCTION ON HERPESVIRUSES

Michael R. Sutherland

**Thesis submitted to the Department of Biochemistry in partial fulfillment of
the requirements for the degree of Master of Science**

**University of Ottawa
Ottawa, Ontario, Canada
January, 1998**

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ABSTRACT

Herpesviruses have been previously correlated to vascular disease and shown to cause thrombogenic and atherogenic changes to host cells. The work conducted in this thesis showed that even in the absence of cells, purified cytomegalovirus (CMV) and herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) could initiate thrombin production. Clotting and chromogenic assays showed that purified HSV-1 and HSV-2 provide the procoagulant phospholipid (proPL) necessary for assembly of factors Xa (FXa) and Va (FVa) into prothrombinase, which is responsible for generating thrombin. These findings are consistent with earlier CMV studies. These observations were confirmed by electron microscopy and flow cytometry using FVa and annexin V, respectively, as proPL-specific probes. CMV, HSV-1 and HSV-2 each had the ability to facilitate FXa generation from the inactive precursor factor X (FX), but only when factor VIII/VIIIa and Ca^{2+} were present. Monoclonal antibodies specific for the coagulation initiator, tissue factor (TF), inhibited this FX activation and furthermore enabled identification of TF antigen on each virus type by electron microscopy and flow cytometry. Collectively, these data show that CMV, HSV-1 and HSV-2 can initiate the generation of thrombin by having essential proPL and TF activities on their surface. Unlike the normal cellular source, the viral activity is constitutive and therefore not restricted to sites of vascular injury. Thus cell-independent thrombin production may be the earliest event in vascular pathology mediated by herpesviruses.

Dedication

When the battles are forgotten, and
the galleon is dust, the crew swims
united through the timeless waters.

For Mom, Jean and Wendy: though separated
we'll always be together

Acknowledgments

Over the past couple of years there have been several people who have contributed to the completion of this project. These include in no particular order: Bea Valentine and Andrew Vaillant for patience in showing me the EM way; Rod Nichols at the University of Ottawa, Electron Microscopy Unit, and Steve Doyle at the University of Toronto, Microscopy Imaging Laboratory, for assistance with electron microscopy; Garry Kessler for protein purification and technical assistance; Dr Réal Lemieux for encouragement and support; Drs Pierre Neuenschwander and James Morrissey for discussions on tissue factor; Dr Fraser Wright for helpful thoughts and suggestions; Dr Henriette Leenknecht for technical assistance, comic relief and for everything she didn't tell; everyone at the Cross; the crew for necessary diversions; and most of all the Collective for their never ending support in every way possible.

A very special thank you goes to Tina Raynor for all her technical assistance, helpful criticism, constant enthusiasm, and rejuvenation of essence.

And finally, Dr Ed Pryzdial. Thanks for the never ending support, knowledge, optimism and motivation to go on. And of course, DQ and the occasional depletion of essence. Thanks

Table of Contents

	Page
Abstract	ii
Dedication	iii
Acknowledgments	iv
Table of Contents	v
List of Figures	xi
List of Abbreviations	xii
1. Introduction	1
1.1 Overview	1
1.2 Normal Thrombin Production	2
1.2.1 Procoagulant Phospholipid	2
1.2.2 Factor X	3
1.2.2.1 Structure	3
1.2.2.2 Activation	5
1.2.3 Prothrombin	8
1.2.3.1 Structure	8
1.2.3.2 Activation	9
1.2.4 Thrombin	10

1.3	Herpesviruses	10
1.3.1	General Characteristics	10
1.3.2	Cytomegalovirus	11
1.3.2.1	Morphology	11
1.3.2.2	Pathology	12
1.3.3	Herpes Simplex Virus	12
1.3.3.1	Morphology	13
1.3.3.2	Pathology	13
1.4	CMV and HSV in Vascular Disease	14
1.4.1	Induction of Thrombogenic Changes to Host Cells	14
1.4.2	Induction of Atherogenic Changes to Host Cells	15
1.5	Rationale	17
2.	Methods	18
2.1	General	18
2.1.1	Reagents	18
2.1.2	Virus Preparation	18
2.1.3	Protein Preparation	19
2.2	Procoagulant Phospholipid-Dependent Experiments	20
2.2.1	Functional Detection	20

2.2.1.1	Clotting Assay	20
2.2.1.2	Chromogenic Assay	20
2.2.2	Direct Detection	21
2.2.2.1	Electron Microscopy	21
2.2.2.2	Flow Cytometry	22
2.3	FXa-Dependent Experiments	23
2.3.1	Functional Activation of FX	23
2.3.1.1	Clotting Assay	23
2.3.1.2	Electrophoretic Detection of FX Activation	23
2.3.1.3	Chromogenic Assay	24
2.3.2	Direct Detection	25
2.3.2.1	Electron Microscopy	25
2.3.2.2	Flow Cytometry	25
3.	Results	26
3.1	Evidence of proPL on Herpesviruses	26
3.1.1	Virus proPL-Dependent Fibrin Detection	26
3.1.2	Chromogenic Detection of Viral proPL-Dependent Thrombin Production	26
3.1.3	Immunogold Detection of proPL on the Herpesviruses Surface	28

3.1.4	Flow Cytometric Detection of proPL on the Herpesviruses Surface	31
3.2	Activation of FX on Herpesviruses	31
3.2.1	Virus-Dependent FXa Generation and Clot Formation	33
3.2.2	Activation of ¹²⁵ I-FX Followed by Electrophoresis	33
3.2.3	Antibody-Mediated Inhibition of TF Activity	36
3.2.4	Immunogold Detection of TF Antigen on Herpesviruses	38
3.2.5	Flow Cytometric Confirmation of TF Antigen on Herpesviruses	40
4.	Discussion	42
4.1	Thrombin Production on Herpesviruses	42
4.2	Possible Effects of Thrombin Produced on Herpesviruses	48
4.2.1	Infection	48
4.2.1.1	Attachment to the Host Cell Surface	49
4.2.1.2	Penetration of the Host Cell Membrane	50
4.2.1.3	Transcription of the First Virus Genes	51
4.2.2	Vascular Disease	52
4.3	Connection Between Other Viruses and proPL	53
4.4	Future Studies	54
4.4.1	Determine if Other Thrombogenic Mechanisms Exist On Herpesviruses	54

4.4.2 Determine the Role of Thrombin Produced on the Virus 54

5. References 56

List of Figures

Figure		Page
1	Structure of FX	4
2	Initiation of thrombin production	7
3	Functional demonstration of viral proPL by clotting assay	27
4	Functional demonstration of viral proPL by clotting assay	29
5	Direct demonstration of proPL on herpesviruses by immunogold electron microscopy	30
6	Direct demonstration of viral proPL by flow cytometry	32
7	Virus-dependent generation of FXa clotting activity	34
8	Virus-dependent proteolytic activation of FX	35
9	Inhibition of virus-dependent FX activation with anti-TF mAb	37
10	Direct demonstration of TF antigen on herpesviruses by immunogold electron microscopy	39
11	Direct demonstration of TF antigen on herpesviruses by flow cytometry	41
12	Model for the thrombogenic mechanisms identified on herpesviruses	44
13	Cell modulation by herpesvirus-produced thrombin	47

List of Abbreviations

Alphabetical List

AnII, annexin II

AnV, Annexin V

Ca²⁺, calcium

CMV, cytomegalovirus

FITC, fluorescein isothiocyanate

FV, coagulation factor V

FVa, activated coagulation factor V

FVII, coagulation factor VII

FVIIa, activated coagulation factor VII

FX, coagulation factor X

FXa, activated coagulation factor X

gC, glycoprotein C

HBS, hepes buffered saline

HSV, herpes simplex virus

HSV-1, herpes simplex virus type 1

HSV-2, herpes simplex virus type 2

IE, immediate early gene product

IgG, immunoglobulin G

kDa, kilodaltons

mAb, monoclonal antibody

proPL, procoagulant phospholipid

PS, phosphatidylserine

S-2238, H-D-Phe-Pip-Arg-p-nitroaniline dichloride

TF, tissue factor

UV, ultraviolet

vp, virus particle

vWF, von Willebrand factor

1. INTRODUCTION

1.1 OVERVIEW

Blood coagulation involves a biological amplification system in which there is sequential activation by proteolysis of circulating precursor enzymes and cofactors. The ultimate result is the generation of thrombin which is responsible for the deposition of an insoluble fibrin network and modulation of cell function at the site of vascular injury. The operation of this enzyme cascade requires local concentration of circulating coagulation factors. Blood contains all the necessary ingredients to form a clot, lacking only the triggering mechanism. The initiating signals are supplied by the cells at the site of vascular injury when the sub-endothelium is exposed, allowing plasma clotting factors to come into contact with procoagulant phospholipid (proPL) and tissue factor (TF). The former provides a surface on which the activation of coagulation enzymes occurs, while the latter is an initiating cofactor. The exposure of both proPL and TF is highly regulated and occurs only at sites of vascular damage. This prevents possible development of occlusive thrombi and ensures that the hemostatic balance between coagulation and the converse process, fibrinolysis, is maintained.

Herpesviruses such as cytomegalovirus (CMV), herpes simplex virus type 1 (HSV-1) and herpes simplex virus type 2 (HSV-2) are highly prevalent and are known to transform endothelial cells, which line all blood vessels, from a resting

noncoagulant to a procoagulant form. Consequently, these viruses have been linked to vascular disease. A related characteristic is that herpesviruses possess the ability to form persistent latent infection in host cells. Reactivation of these viruses and expression of viral gene products have been postulated to be initiating factors in persistent vessel reinjury leading to atherosclerosis. Many of the changes to the cell caused by these viruses can be attributed to the production of thrombin at the cell surface.

While the ability of herpesviruses to alter the thrombogenic and atherogenic state of the host cells has been well documented, the participation of virus independent of cells has not been studied. The purpose of this thesis is to investigate surface constituents of CMV, HSV-1 and HSV-2 that may directly contribute to thrombin production even before cellular procoagulant responses are triggered.

1.2 NORMAL THROMBIN PRODUCTION

1.2.1 Procoagulant Phospholipid

To be considered procoagulant, a cell membrane must express proPL of which phosphatidylserine (PS) is considered to be the most important (31,95,102,134). The function of proPL is to increase the reaction rate of coagulation enzymes. This is accomplished by: 1) concentrating the enzyme,

cofactor and substrate by recruitment of clotting proteins from blood (79,84,104); 2) assuring the protein components are maintained in the correct orientation for reactions to occur (58,143); 3) stabilizing the interaction with non-enzymatic cofactors (2,3,81,113); and 4) preventing the inactivation of enzymes by circulating anticoagulants (95). Therefore, the availability of accessible proPL is essential for the assembly of coagulation enzyme complexes.

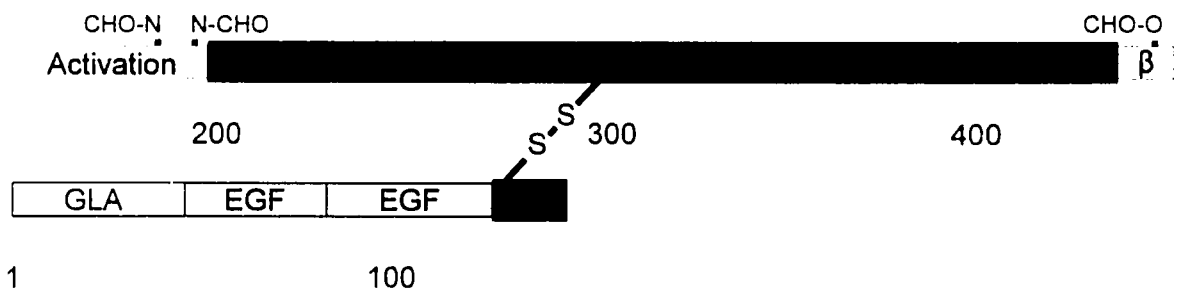
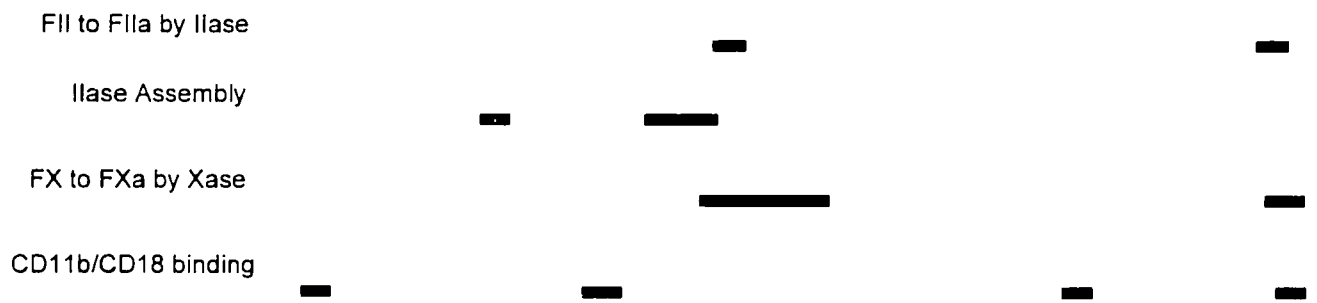
Another very important function of proPL is the localization of thrombin production. When vascular damage occurs, the exposure of the subendothelium or the release of cell agonists induces a rearrangement of cell membrane phospholipid. This results in a transbilayer "flip" of proPL from the inner to the outer face of cells adjacent to the site of injury (13,19,20,84,99,109,132,151). To prevent thrombin generation at all other locations, cells maintain a highly asymmetric distribution of membrane phospholipid with proPL exclusively on the cytoplasmic side. The exposure of proPL is therefore a very important hemostatic control mechanism and is highly regulated. Endothelial cells, activated platelets and monocytes are the most important physiological sources of proPL (88,133,144).

1.2.2 Factor X

1.2.2.1 Structure

Factor X (FX) circulates in plasma as a zymogen. As shown in Figure 1 pg4

FIGURE 1 Structure of FX. FX circulates in plasma as a two chain zymogen. The light chain of FX contains the gamma carboxy glutamic acid domain (GLA), that functions in proPL binding and two epidermal growth factor like domains (EGF). The light chain is linked to the heavy chain by a single disulfide bond. The functional regions of the heavy chain are indicated. Upon activation to FXa, a cleavage occurs in the carboxy terminal of the heavy chain and subsequent release of an activation fragment (Activation).



it is composed of a light chain (16 kDa) and a heavy chain (42 kDa) that are linked by a disulfide bond. The light chain contains vitamin K-dependent posttranslational modifications of glutamic acid to gamma carboxyglutamic acid (gla). It is the gla-containing domain that functions in binding to proPL. This property is similar for all other vitamin K-dependent coagulation proteins such as prothrombin and factor VII (FVII). The heavy chain of FX contains the catalytic domain and functions in substrate and cofactor binding. FX must be converted to an active form, factor Xa (FXa), by a tenase complex before it can participate in clotting. This involves the cleavage of the Arg 52-Ile 53 of the COOH-terminal heavy chain and subsequent release of a 52 amino acid activation fragment.

1.2.2.2 Activation

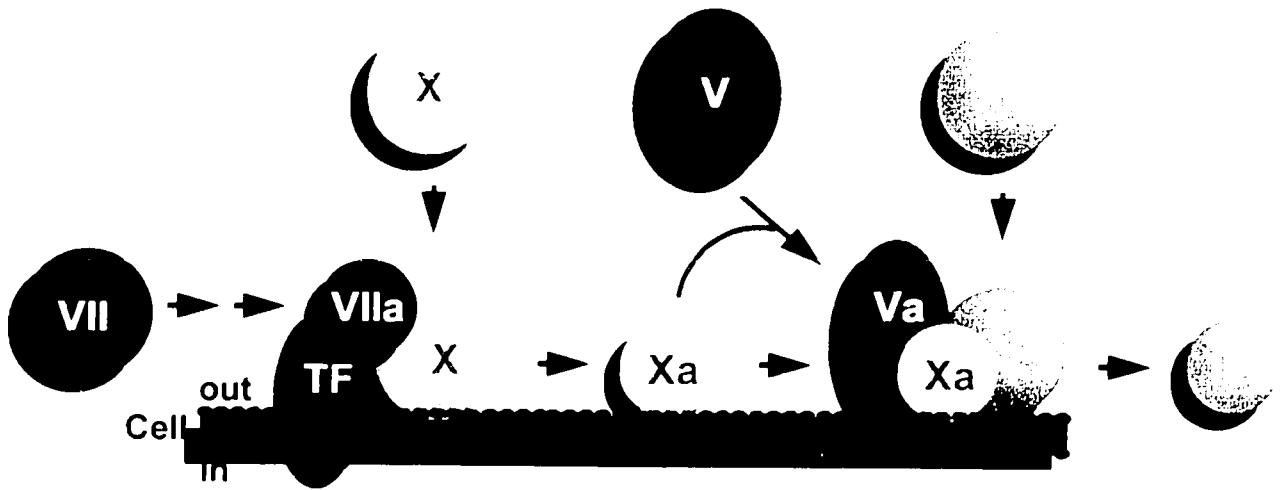
Several complexes that convert FX to FXa are known. These FX-activating complexes are termed tenase. The tenase responsible for initiation of the coagulation pathway consists of the serine protease factor VIIa (FVIIa) bound to its specific cofactor tissue factor (TF) (14,101). The FVIIa precursor, FVII, is a single chain molecule of approximate 50 kDa. Its proteolytic activation is likely contributed to by several enzymes of which one is a cellular protein called hepsin. The activity of FXa or autoproteolysis may also be involved. The activation reaction is due to the cleavage of the peptide bond following Arg 152. The two resulting chains are held

together by a disulfide bond with the catalytic domain contained in the heavy chain.

The normal initiation of thrombin production is triggered by vascular damage and is strictly limited to neighboring cells where the transmembrane protein TF and proPL become accessible to plasma clotting factors (see FIGURE 2, pg 7). The role of TF is to accelerate FVIIa. Based on work using model vesicles, the FVIIa/TF interaction and cofactor activity of TF were shown to be phospholipid independent (122). However, the association of FX with PS-containing vesicles was observed to enhance the overall V_{max} of the reaction with a modest decrease in K_m (70). TF (35 kDa) is normally found only in the arterial adventitia. However, endothelial cells, monocytes, smooth muscle cells and fibroblasts can express TF after stimulation by various cell antagonists, one example of which is thrombin (15,109). Thus, initiation of coagulation is dependent upon the availability of TF on the cell surface for generating the first FXa molecules.

While the TF/FVIIa complex is considered the initiating tenase, a second tenase is necessary to amplify the coagulation pathway. This second tenase is a Ca^{2+} -dependent complex between the protease factor IXa (FIXa), its specific cofactor VIIIa (FVIIIa) and proPL. The platelet membrane is considered the most important source of proPL for assembly of FIXa/FVIIIa complexes (4). In blood, FVIIIa circulates as its inactive precursor, factor VIII(FVIII), bound to von Willebrand factor. FVIII cannot assemble into tenase complexes until it is released by

FIGURE 2 Initiation of thrombin production. The initiation of thrombin (IIa) production is triggered by exposure of the transmembrane protein tissue factor (TF) and procoagulant phospholipid, proPL, (light colored head groups). Factor VII (FVII) becomes activated (FVIIa) and combines with TF. The TF/FVIIa complex then binds factor X (FX) and activates the zymogen to FXa. The prothrombinase complex is then formed when FXa and its cofactor, factor Va (FVa), associate through proPL dependent interactions. This complex will convert prothrombin (II) to the potent cell modulator and biological effector of coagulation, thrombin.



proteolytic conversion to FVIIIa. The initial generation of FVIIIa activity is mediated by FXa, but is more efficiently generated by thrombin.

An additional FX-activating pathway also occurs on monocytes. This route is ADP- or ionomycin-inducible (6-8,10) and involves FX-binding to the type 3 complement receptor (CR3) and an as yet unidentified FX-activating enzyme. CR3 is a member of the integrin family of cell surface adhesion proteins, composed of an α M-chain (CD11b, 155 kDa) and a β 2-chain (CD18, 94 kDa) in a Ca^{2+} -dependent complex (11,12,32).

Important to the current research is that a tenase cofactor encoded by the HSV-1 genome has been identified (9,38). The 100-130 kDa HSV-1 encoded glycoprotein C (gC) expressed on the surface of infected endothelial cells has been shown to have FX binding and activating properties that are analogous to those of CR3 (38). gC also exists on the virus surface and has been shown to participate in the initial HSV-host cell interaction (131) and in the evasion of complement-mediated immune clearance (43). We therefore speculated that FX activation could occur directly on the HSV surface via gC.

1.2.3 Prothrombin

1.2.3.1 Structure

Prothrombin consists of a single polypeptide chain that is activated to the

potent cell modulator thrombin. This proteolysis is catalyzed by the prothrombinase enzyme complex. During the activation, prothrombin is cleaved at Arg 273-Thr 274 and at Arg 323-Ser 324 to yield a pro fragment, which contains the proPL-binding gla-domain, and thrombin. Thrombin is a two chain enzyme composed of an amino terminal "A" chain (6 kDa) and a carboxy terminal "B" chain (31 kDa) which remain covalently associated through a single disulfide bond. The catalytic triad common to all serine proteases is located in the B chain of the thrombin molecule.

1.2.3.2 Activation

The principal role of FXa is to activate prothrombin to thrombin. For this to occur, FXa must first associate with the cofactor Va (FVa) and proPL in the presence of Ca^{2+} (see FIGURE 2, pg 7). Together, FXa, FVa and proPL constitute the prothrombinase complex, which confers a 280,000 fold increase in enzymatic activity to FXa (72,103). The assembly of prothrombinase involves interactions between FXa and FVa (113); FVa and a proPL-containing membrane ; and, FXa and a proPL-containing membrane (71,102), each having an element of Ca^{2+} -dependence (45,73,100,102,113). Like FVIIIa, FVa circulates as an inactive precursor (FV) and is proteolytically activated by thrombin or FXa.

1.2.4 Thrombin

Thrombin is the final enzyme produced in the blood coagulation cascade. It is responsible for the generation of insoluble fibrin clot and feedback-amplification of coagulation enzyme activity. The latter is partly accomplished through rapid activation of the procofactors FVIII and FV. Of equal physiological significance is the ability for thrombin to function as a very potent cell modulator that can induce important intracellular and extracellular regulatory processes (34,46,83). Thrombin-sensitive cells and some of their responses to thrombin include: platelets (76,89), which express proPL and secrete other cell agonists; endothelial cells (38,49,87,110,124,136), that express TF, proPL and integrin-type receptors and secrete platelet activating factor; leucocytes (61,62,98), which express TF and proPL and adhere to the endothelial integrins and facilitate inflammation; and smooth muscle cells, which express TF and proliferate after treatment with very low concentrations of thrombin (50,89). Thrombin also has an important role in the anticoagulant pathway by activating protein C (36) and has been demonstrated to be involved in the regulation of fibrinolysis (16).

1.3 HERPESVIRUSES

1.3.1 General Characteristics

A typical herpesvirion consists of a core containing linear double-stranded

DNA. The DNA is encapsulated in an icosahedral capsid of approximately 100 nm. This structural feature contains 162 capsomeres which are a mixture of pentameric and hexameric arrangements that are characteristic of all herpesviruses. The capsid is then surrounded by a proteinaceous layer termed the tegument. This asymmetric structure between the capsid and envelope has no distinctive features and varies in thickness. The viral tegument in turn is covered by an envelope which contains lipid solely derived from the host-cell and proteins that are encoded by both the virus and host genome. The herpesvirus contains spikes which are virus encoded glycoproteins, the number and relative amounts varying among the virus types.

All herpesviruses share four significant biological properties. These include: 1) a large array of enzymes specified by the virus that are involved in DNA synthesis, nucleic acid metabolism and processing of proteins; 2) the synthesis of viral DNA and capsid assembly in the nucleus with subsequent envelopment through cellular membranes; 3) the production of infectious virus is accompanied by the irreversible destruction of the cell; and 4) the ability to remain latent in their hosts.

1.3.2 Cytomegalovirus

1.3.2.1 Morphology

CMV is a member of the beta herpesviridae family (117,118). This virus type

characteristically produces enlarged cells with intranuclear inclusions and are known to have a long reproductive cycle. The DNA core potentially encodes greater than 200 gene products (26). Mature virions are large, having an average diameter of approximately 150-200 nm. During the viral maturation process two non-infectious forms of CMV may also be produced. These include dense bodies (DB) and non-infectious enveloped particles (NIEP). The DB and NIEP lack viral DNA and exhibit different morphological characteristics when compared to infectious virions by electron microscopy (59).

1.3.2.2 Pathology

Evidence for CMV exposure is detectable in at least 50% of adult North Americans. In normal individuals CMV infection is usually subclinical, although it has been found to account for some mononucleosis-like symptoms (67). Severe CMV-related pathology occurs in patients who are immunocompromised either therapeutically (i.e. transplant and radiotherapy recipients) or biologically (i.e. AIDS) (33,52,92). Disseminated CMV infection is a prevalent complication as a result of this extreme immunodeficiency and increases the morbidity and mortality with primary and recurrent infections.

1.3.3 Herpes Simplex Virus

1.3.3.1 Morphology

Herpes simplex virus types 1 (HSV-1) and -2 (HSV-2) are members of the alpha herpesvirus family. These viruses have a short, rapid reproductive cycle with complete destruction of infected cells. The HSV genome is approximately 150 Kbp which has been estimated to encode 75 gene products. During mature virion formation, three capsid types may result. These capsids differ by: 1) the presence of DNA; 2) the absence of DNA; and 3) the site of envelopment. Each of the three capsid types are readily distinguishable by electron microscopy.

1.3.3.2 Pathology

Herpes simplex virus infections occur worldwide, with humans as the only natural reservoir. There are two distinct serotypes: HSV-1 which is transmitted mainly by contact with oral secretions and; HSV-2 which is transmitted by contact with genital secretions. 80-90% of the population have antibodies to HSV-1 by the age of 50, while 20% are positive for antibodies to HSV-2 by the same age (141). After primary infection, the virus persists in the form of an asymptomatic latent infection. Fever blisters are the most common manifestation of HSV-1 reactivation, while recurrent genital herpes often follows HSV-2 infection. No single defect in the immune response has been identified as responsible for HSV persistence and recurrence in an immunocompetent host.

1.4 CMV AND HSV IN VASCULAR DISEASE

1.4.1 Induction of Extracellular Thrombogenic Changes to Host Cells

CMV (135,136) and HSV (38,39,140) are known to convert resting vascular endothelial cells from a noncoagulant to a procoagulant state. Various thrombogenic changes have been identified in response to HSV that explain the viral influence on cellular procoagulant activity. These include: expression of TF (138); expression of HSV-1 gC (9); and rearrangement of the membrane phospholipid (140). The latter may result in the exposure of proPL on the cell surface allowing the more efficient formation of coagulation enzyme complexes. Alternatively, HSV-induced cellular changes that reduce the inherent anticoagulant properties of cells have also been observed. Among these are: decreased prostacyclin secretion (140), a platelet activation inhibitor; reduced expression of thrombomodulin (138), a cofactor within the protein C-dependent anticoagulant pathway; and reduced synthesis of heparan sulfate proteoglycan (63), a cofactor within the serpin-dependent anticoagulant pathway. Definitive molecular changes in response to CMV infection which result in a procoagulant phenotype have not been studied as extensively. Two possible changes due to CMV include the secretion of vWF (24) and rearrangement of the membrane phospholipid have been reported (136). These factors therefore cause the hemostatic balance between thrombogenic and anticoagulant pathways associated with the cell to be altered by

the virus. The ultimate result in all cases is to favor thrombin production.

1.4.2 Induction of Atherogenic Changes to Host Cells

Atherogenesis is believed to follow a response to endothelial injury, which exposes the underlying arterial smooth muscle cells to stimuli causing them to proliferate excessively. Considerable advancement has been made in understanding circulating lipids and abnormal lipid metabolism, and their role in atherosclerosis. However, the causative agent still remains unknown while increasing attention is focusing on the cellular abnormalities of lesions in the vascular wall.

There are several lines of evidence that support a CMV and HSV link to atherosclerosis: 1) CMV and HSV genetic material has been detected within the endothelial and smooth muscle cells of atherosclerotic tissue (1,18,30,47,51,53,93,94); 2) a strong correlation of active CMV infection to an accelerated form of atherosclerosis in immunosuppressed organ transplant recipients has been documented (42,56,57,147); 3) CMV infection has been shown to be a strong risk factor for restenosis after angioplasty (35,150); and 4) CMV has been linked to vascular thickening (106).

Since aberrant smooth muscle cell proliferation and recruitment of inflammatory cells are central features of atherosclerotic plaque development

(60,94), the ability to promote the generation of thrombin as a cell stimulant may link subclinical CMV or HSV infection to atherosclerosis (90,92,94,138). The virus-mediated generation of thrombin may also explain the observation of fibrin deposits in the microvasculature of mucosal lesions caused by HSV (77,90) and the development of disseminated intravascular coagulation in neonates with severe HSV infection (90). While the clinical data are arguably circumstantial, a distinct cause-and-effect relationship was established in avian and rodent models where viral infection induced atherosclerosis and thrombosis (40,41).

Important advancements have recently been made in understanding the biochemical mechanisms connecting CMV exposure and the expression of genes that have been incorporated into the host genome (i.e. latent infection) to atherosclerosis. The first example involves the CMV gene product, IE84, which is capable of associating with the tumor suppressor protein, p53, and inducing smooth muscle cell proliferation (126). Work from the same laboratories has also demonstrated that the CMV gene product, IE72, triggers the synthesis and expression of a "scavenger" receptor for oxidized low density lipoprotein (LDL) deposition, which is an early event in atherosclerosis (23). The cumulative biochemical evidence provides a molecular explanation for the clinical studies.

1.5 RATIONALE

In a previous study from our laboratory, accessible proPL was identified on CMV (114). It presented a novel role for the CMV envelope in pathology by suggesting that the control mechanism set up by the cells to restrict the expression of proPL specifically to areas of vascular injury is lost. Therefore, CMV can continuously present proPL to clotting factors in plasma, bypassing the normal regulatory pathway. This implies that herpesviruses could contribute to vascular disease through the production of thrombin, even before affecting the host cell.

The purpose of the current work was to determine if the herpesviruses implicated in vascular disease can participate in thrombin production independent of cells. More specifically, the following goals were undertaken: 1) Determine whether, like CMV, proPL exists on the surface of HSV-1 and HSV-2, and 2) Determine whether FX activation is supported on the CMV, HSV-1 and HSV-2 surfaces.

2. Methods and Materials

2.1 GENERAL

2.1.1 Reagents

H-D-Phe-Pip-Arg-p-nitroaniline dichloride ((S-2238) Chromogenix), ethylenediamine tetraacetic acid (EDTA), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes) were from Sigma. All experiments were conducted in Hepes buffered saline (HBS; Hepes (20 mM), NaCl (150 mM) pH 7.2).

2.1.2 Virus Preparation

Human foreskin fibroblasts (HFF) were grown to confluence in Basal Medium Eagle (Gibco) supplemented with 5% bovine calf serum, 2 mM glutamine and 20 µg/ml gentamycin. The monolayers were inoculated with CMV (strain AD 169) at a multiplicity of infection of 0.001, and harvested from the supernatant when a full cytopathic effect was apparent. The supernatant was clarified by centrifugation at 700 x g and the virus was pelleted at 23,000 x g. The virus pellet was resuspended in HBS and subjected to ultracentrifugation at 200,000 x g through a 40% potassium tartrate / 20% glycerol gradient. The band corresponding to virions was separated from those due to other particles (non infectious enveloped particles and dense bodies) (114,145). Purified HSV-1 (MacIntyre strain) and HSV-2 (strain G) were obtained commercially (Advanced Biotechnologies Inc.). All virions were quantified

and evaluated for purity by electron microscopy using negative staining as previously described (114,145). Briefly, virions were adsorbed onto carbon/formvar grids in HBS containing BSA (0.01%). Phosphotungstic acid (2%) was then added and excess sample was blotted off the grid. A known amount of similarly sized polystyrene latex spheres (EM Sciences) were added to aid in the quantification. Less than 10% of particles in the virus preparations was attributed to cellular debris.

2.1.3 Protein Preparation

Human coagulation proteins FVIIa, FVII and FVa (Haematologic Technologies) and prothrombin (Enzyme Research Laboratories) were obtained commercially. Human FX and FXa were prepared in-house as previously described (112). FX was radioiodinated on ice using Iodogen (Pierce) according to manufacturers instructions. The specific activity was typically 1.2×10^5 cpm/ μg FX. Annexin V ((AnV), kindly supplied by Dr. T. Yokoyama (Kowa Co. Ltd., Japan) was labeled with fluorescein isothiocyanate (FITC), as previously described (68). Using the extinction coefficients for AnV and FITC (130), the conjugate (F-AnV) was purified to a molar ratio of 1 AnV : 2 FITC by FPLC on a Mono Q column developed with a salt gradient in HBS containing EDTA (1 mM).

2.2 PROCOAGULANT PHOSPHOLIPID-DEPENDENT EXPERIMENTS

2.2.1 Functional Detection

2.2.1.1 Clotting Assay

In manual tilt clotting assays, CMV (3.8×10^9 virus particles (vp)/ml), HSV-1 (1.7×10^8 vp/ml) and HSV-2 (6.6×10^7 vp/ml) were incubated with FX deficient plasma (Sigma) for 1 minute at 37°C. At this point FXa (20 nM) was added and clot production was initiated with Ca^{2+} (2 mM). The functional amount of proPL was determined using a standard curve based on synthetic small unilamellar vesicles (PCPS) composed of 75% phosphatidylcholine (PC) and 25% PS that were prepared by centrifugal fractionation and quantified by acid hydrolysis followed by a phosphorous colorimetric assay, as previously described (72). The standard curve was obtained by incubating varying amount of PCPS (0.001-100 μM) in FX deficient plasma for 1 minute at 37°C. FXa (20nM) was added and clot formation was initiated with Ca^{2+} (2mM) and clotting times recorded. An amount of virus was chosen to produce activity within the linear region of the standard curve. Values were corrected for background activity generated in the absence of virus.

2.2.1.2 Chromogenic Assay

CMV (7.2×10^7 vp/ml), HSV-1 (6.9×10^7 vp/ml) and HSV-2 (6.6×10^7 vp/ml) were incubated for 30 minutes with FXa (5 nM), FVa (5 nM) and prothrombin (1.4

μM). The formation of thrombin was initiated by the addition of Ca^{2+} (2 mM). Thrombin generation was monitored by following the rate of cleavage of the specific chromogenic substrate S-2238 in a kinetic microplate reader (Vmax, Molecular Devices). The amount of proPL was determined using a standard curve constructed by incubating PCPS (0.001-10 μM) for 30 minutes with FXa (5 nM), FVa (5 nM), prothrombin (1.4 μM) and Ca^{2+} (2 mM). Cleavage of S-2238 was once again monitored and correlated to thrombin generation. Values were corrected for residual activity in the absence of phospholipid.

2.2.2 Direct Detection

2.2.2.1 Electron Microscopy

For detection of proPL and direct FVa binding, virions were incubated with FVa at a final concentration of 50 ng/ml for 30 minutes at 4°C in HBS plus 2 mM Ca^{2+} (HBS/Ca). The virus was then adsorbed onto carbon/formvar coated grids. After washing 5x with HBS/Ca/0.1% fish gelatin, the grids were treated with anti-FVa-heavy subunit mAb (Haematologic Technologies, Inc. #AHV-5146), or as a negative control with anti-actin mAb (Sigma #A-2547), both at 50 ng/ml for 45 minutes at 22°C. Washing was repeated, followed by incubation with 10 nm gold-conjugated secondary antibody (goat anti-mouse IgG (Sigma)), 100 ng/ml for 45 minutes at 22°C and another round of washing. Negative staining using 2%

phosphotungstic acid was then conducted as described (114,145).

2.2.2.2 Flow Cytometry

Prior to analysis by flow cytometry, purified CMV, HSV-1 and HSV-2 were inactivated for 1 hour on ice at a distance of 5 cm from a UV germicidal lamp (GE 03OT8, 30W). UV inactivation was confirmed by viral formation plaque assays using HFF monolayers as host cells (145). Three methods were used to ensure the identity of virus particles detected by flow cytometry. First, 0.1 μm microspheres (Fluospheres, Molecular Probes) were used to define the boundaries of forward scattering expected for the similarly sized virus particles. Second, varying concentrations of purified virions were run to indicate the location of the virions on the side versus forward scatter dot-plot relative to background. Third, CMV was directly detected by binding of fluorescein-conjugated glycoprotein B-specific (CMVB-1) mAb (Dr. B. Brodeur, Laboratory Centre for Disease Control, Canada). These procedures showed that the side-to-side scatter pattern was relatively homogenous, which allowed us to collect data ungated for all flow cytometry. The identification of viral particles on the flow cytometer and flow cytometry experiments to detect proPL on viruses were conducted by Ms. C. Raynor from our laboratory.

To detect proPL, UV-inactivated CMV (2.3×10^8 vp), HSV-1 (1.3×10^7 vp) and HSV-2 (5.3×10^5 vp) were incubated with F-AnV (0.35 μM) in the presence of

Ca²⁺ (5 mM) or as a negative control in EDTA (5 mM). After incubation for 1 hour at room temperature, samples were diluted to 500 µl in the appropriate Ca²⁺- or EDTA-containing Hepes (20 mM), NaCl (150 mM), pH 7.4 (HBS). Data were immediately acquired using a Becton Dickinson (FACSolv) flow cytometer and analyzed using Lysys II software.

2.3 FXa-DEPENDENT EXPERIMENTS

2.3.1 Functional Activation of FX

2.3.1.1 Clotting Activity

In manual tilt assays, CMV (3.8×10^9 vp/ml), HSV-1 (1.7×10^8 vp/ml) and HSV-2 (6.6×10^7 vp/ml) were incubated with excess PCPS (300 µM) in either FX or FX/VII deficient plasma (Sigma) for 1 minute at 37°C. FX (200 nM) was added and clot formation was initiated with Ca²⁺ (2 mM). The amount of FXa generated was determined using a standard curve of clotting times obtained when purified FXa (0.001-10 µM) was incubated in either FX or FX/VII deficient plasma with Ca²⁺ (2 mM). As before, the amount of virus was adjusted to produce activity within the linear region of the standard curve. Values were corrected for residual FXa activity produced in the absence of virus.

2.3.1.2 Electrophoretic Detection of FX Activation

¹²⁵I-labeled FX (100 nM) was incubated with CMV (1.4 x 10⁸ vp/ml), HSV-1 (6.9 x 10⁷ vp/ml), HSV-2 (6.6 x 10⁷ vp/ml) or without virus in the presence of combinations of FVIIa (1 nM) and Ca²⁺ (2 mM) at 37°C for 30 minutes. The amount of virus was chosen to produce comparable amounts of FX-activation based on the chromogenic assay. The reaction mixtures were then subjected to SDS-PAGE using a 12% acrylamide gel (75) under reducing conditions (2% β-mercaptoethanol). The electrophoretic pattern of FX under each condition was visualized by autoradiography on Kodak X-OMAT film.

2.3.1.3 Chromogenic Assay

CMV (7.2 x 10⁷ vp/ml), HSV-1 (6.9 x 10⁷ vp/ml) and HSV-2 (6.6 x 10⁷ vp/ml) were incubated with or without each of FX (100 nM), FVIIa (1 nM) and Ca²⁺ (2 mM) for 5 minutes at room temperature. FVa (3 nM) and prothrombin (1.4 μM) were then added and the reaction mixture incubated for a further 25 minutes. Thrombin generation was measured using S-2238. Standard curves were prepared under the same conditions with known amounts of FXa added (0-100 nM). The complete reaction mixture was taken as 100% activity. To determine whether TF was involved in the generation of FXa activity by viruses, CMV and HSV-1 and HSV-2 were preincubated with 40 μg/ml of the inhibitory anti-TF mAb (American Diagnostica, product # 4508) or non-immune mouse IgG (Sigma) at 4°C for 2 hours.

2.3.2 Direct Detection of TF

2.3.2.1 Electron Microscopy

Virions were evaluated for endogenous TF antigen by immunogold staining using a TF-specific primary mAb (American Diagnostica, #4503) and 10 nm gold-conjugated secondary antibody (goat anti-mouse IgG (Sigma)), as previously described (2.2.2.1) for detection of proPL.

2.3.2.2 Flow Cytometry

Prior to flow cytometric analysis UV-inactivated CMV (4.1×10^7 vp), HSV-1 (8.0×10^7 vp) and HSV-2 (7.7×10^7 vp) were incubated for 1 hour at room temperature with either anti-TF mAb (American Diagnostica, product #4503) at a dilution of 1:28, 1:12 or 1:24 respectively, or the same amount of non-immune mouse IgG (Cappel) as a negative control. Bound primary antibody was detected by incubation with goat anti-mouse IgG R-phycoerythrin conjugated secondary antibody (Molecular Probes) at dilutions of 1:110, 1:55 or 1:180, respectively for 1 hour at room temperature. The samples were then diluted in 1 ml filtered HBS and the data were acquired immediately after dilution using an Epics XL flow cytometer (Coulter) and analyzed using Facsolv-software. Controls for flow cytometric detection of virus were as previously described for detection of proPL (2.2.2.1). Flow cytometry experiments to identify TF on the surface of viruses were conducted by Dr. H. Leenknecht from our laboratory.

3. RESULTS

3.1 EVIDENCE FOR proPL ON HERPESVIRUSES

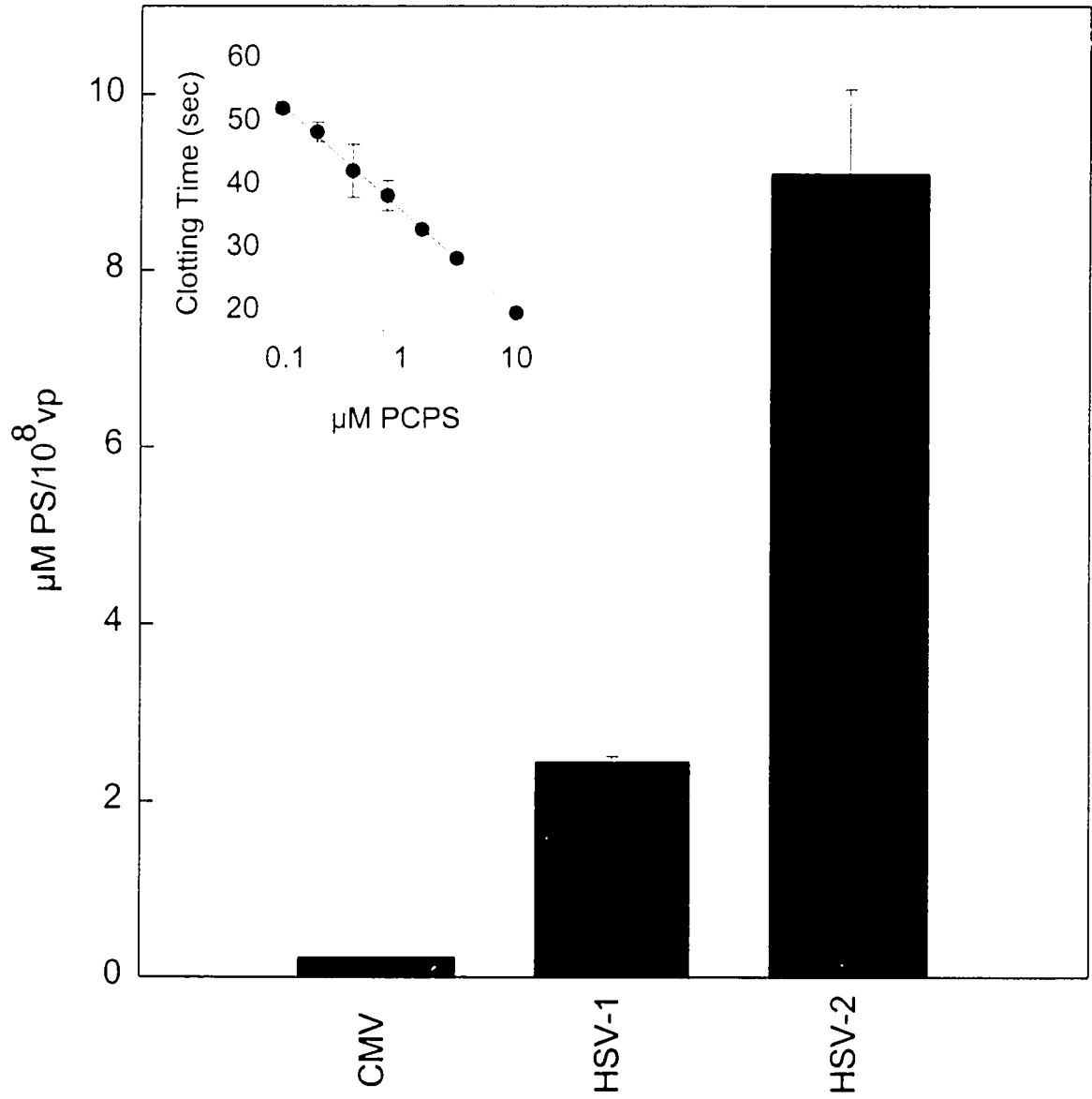
3.1.1 Virus proPL-dependent Fibrin Generation

Previous studies from our laboratory showed that proPL exists on the surface of CMV (114). To determine whether other herpesviruses also exhibit proPL activity on their surface, HSV-1 and HSV-2 were evaluated. Using proPL-dependent tilt clotting assays, CMV, HSV-1 and HSV-2 were included as the sole source of phospholipid. As seen in Figure 3, pg 27, all three viruses reduced the clotting time of FX-deficient or combined FX- and FVII-deficient plasma by at least 30 seconds. Therefore, each virus surface must possess the necessary proPL to allow successful prothrombinase formation and subsequent production of thrombin. These experiments demonstrated that the HSVs had considerably more apparent proPL activity than CMV per virion (CMV, $0.23 \pm 0.01 \mu\text{M proPL}/10^8$ virus particles (vp); HSV-1, $2.4 \pm 0.1 \mu\text{M proPL}/10^8$ vp; HSV-2, $9.1 \pm 1.4 \mu\text{M proPL}/10^8$ vp; n=3).

3.1.2 Chromogenic Detection of Viral proPL-Dependent Thrombin Production

A second functional method was used to further support the presence of proPL on the three enveloped viruses. In this system, virus was again added as the source of proPL but prothrombinase was assembled on the virions using purified

FIGURE 3 Demonstration of viral proPL by a clotting assay. CMV, HSV-1 and HSV-2 were incubated for 1 minute with FX deficient plasma at 37°C. After FXa (20 nM) was added, the reaction was initiated with Ca²⁺ (2 mM) and the clotting times recorded. The amount of corresponding proPL was determined using a PCPS standard curve (inset). (n=3)



FXa and FVa with purified prothrombin as the substrate. The addition of each virus resulted in chromogenic substrate cleavage, corresponding to thrombin generation (Figure 4, pg 29). This method confirmed that all three viruses have accessible proPL activity (CMV, 0.48 ± 0.36 nM proPL/ 10^8 vp; HSV-1, 287.1 ± 70.8 nM proPL/ 10^8 vp; HSV-2, 194.4 ± 58.1 nM proPL/ 10^8 vp; n=4). While the presence of proPL for each virus type was clear and was at least comparable to that expected for activated platelets (114), the two assay methods reproducibly gave different quantitative results. These differences may have arisen from an as yet unidentified component(s) in plasma that influences the apparent proPL activity.

3.1.3 Immunogold Detection of proPL on the Herpesvirus Surface

Having obtained functional evidence, a physical demonstration of proPL on the virus was obtained by immunogold electron microscopy (Figure 5, pg 30). Indicating the presence of proPL, CMV, HSV-1 and HSV-2 bound exogenous FVa, which was detected using a FVa-heavy subunit-specific mAb. The electron-dense spots indicate the recognition of FVa on the virus. Approximately 80% of all virus particles including both lysed ("fried egg"- shaped) and intact (spherical) virus particles were found to bind FVa, whereas no positive particles were found in control experiments using a non-immune primary antibody. Endogenous FV/Va was not detectable (data not shown). Combined with our earlier electron microscopy

FIGURE 4 Demonstration of viral proPL by chromogenic assay. Viruses were incubated for 30 minutes with FXa (5 nM), FVa (5 nM) and prothrombin (1.4 μ M) in the presence of Ca^{2+} (2 mM). The formation of thrombin was quantified using the chromogenic substrate S-2238. The amount of corresponding proPL was determined using a PCPS standard curve (inset). (n=4)

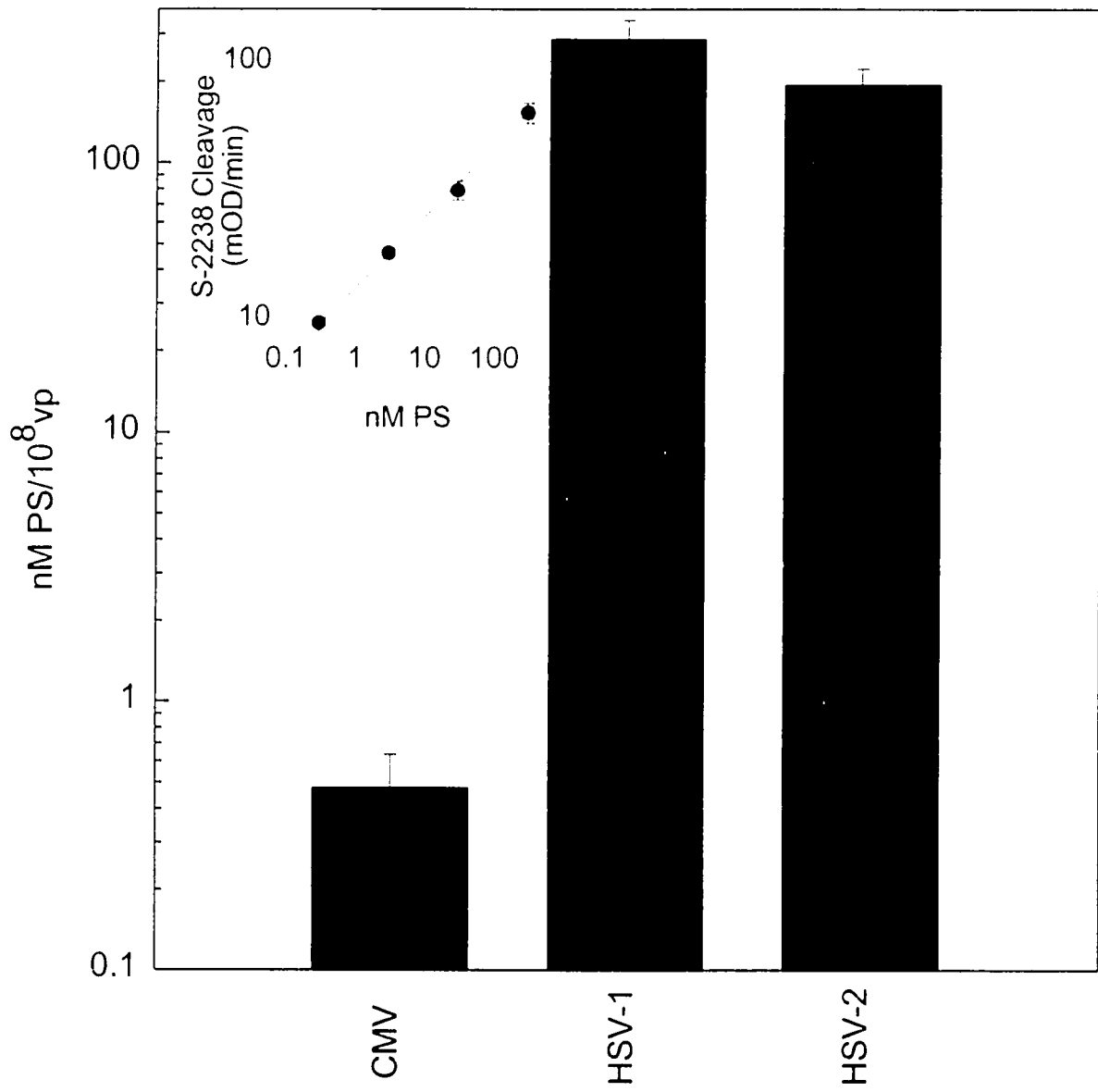
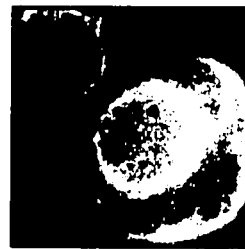


FIGURE 5 Direct demonstration of proPL by electron microscopy. Virions were incubated with FVa at a final concentration of 50 ng/ml for 30 minutes at 4°C. The virus was adsorbed onto carbon/formvar coated grids in HBS containing Ca²⁺ (2 mM) (HBS/Ca). After washing 5x with HBS/Ca/0.1% fish gelatin, the grids were treated with anti-FVa-heavy subunit mAb, or as a negative control with anti-actin mAb, both at 50 ng/ml for 45 minutes at 22°C. Washing was repeated, followed by incubation with 10 nm gold-conjugated secondary antibody at 100 ng/ml at 22°C and another round of washing. The grids were then negative stained with phosphotungstic acid.

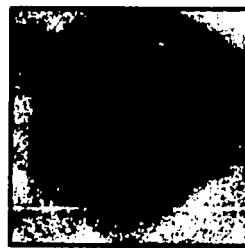
control

anti-FV

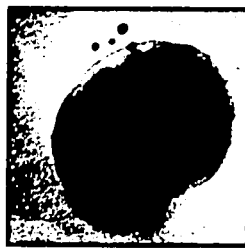
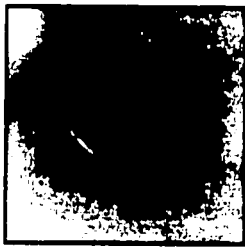
CMV



HSV-1



HSV-2



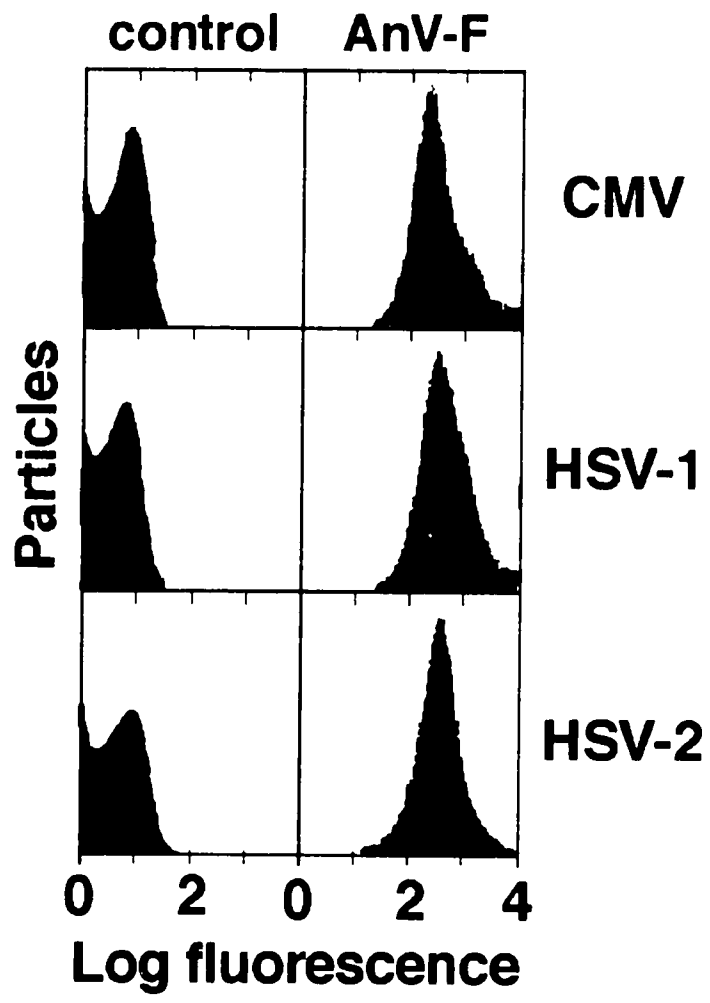
demonstrating FXa-binding to CMV (114), the current data provide convincing evidence that both prothrombinase constituents can assemble directly on virus particles.

3.1.4 Flow Cytometric Detection of proPL on the Herpesvirus Surface

The immunogold data were corroborated by flow cytometry. Annexin V conjugated to fluorescein (F-AnV), which is known to bind anionic phospholipid in a Ca^{2+} -dependent manner (91,128,129), was shown to recognize virus particles. Figure 6, pg 32, shows that all particles present in the CMV, HSV-1 and HSV-2 preparations could specifically bind F-AnV in the presence of Ca^{2+} , but not when a chelator (EDTA) was included as a negative control. Chelation had no significant effect on the side-to-side scatter pattern of any virus preparation. Therefore, the demonstrated proPL activity was associated with the virus particles and not due to the potentially copurifying cellular debris (found to represent <10% of all particles observed by electron microscopy) or by aggregation caused by different divalent cationic conditions. It should be noted that a number of particles were accumulated on the 10^4 channel axis for the CMV and HSV-1 samples containing Ca^{2+} (not shown).

3.2 ACTIVATION OF FX ON HERPESVIRUSES

FIGURE 6 Direct demonstration of viral proPL by flow cytometry. UV-inactivated CMV, HSV-1 and HSV-2 were incubated with AnV-F (0.35 μ M) in the presence of Ca^{2+} (5 mM) or as a negative control EDTA (5 mM). After incubation for 1 hour at room temperature, samples were diluted in the appropriate Ca^{2+} - or EDTA-containing HBS, pH 7.4 and data were immediately accumulated.



3.2.1 Virus-Dependent FXa Generation and Clot Formation

The controlled availability of cell surface proPL and TF is an important regulator of FXa generation. Having established the availability of proPL on CMV, HSV-1 and HSV-2, it was speculated they may also have constituents that can facilitate FX activation. To test this hypothesis, each virus type was incubated with purified FX and either FX-deficient or combined FX- and FVII-deficient plasma, and evaluated for clotting times. No detectable FXa activity was generated when FX/FVII-deficient plasma was used (data not shown). In contrast, Figure 7, pg 34, shows significant FXa activity was produced when FVII was present in the plasma (CMV, 25.2 ± 4.5 nM FXa/ 10^8 vp; HSV-1, 74.7 ± 6.6 nM FXa/ 10^8 vp; HSV-2, 185.5 ± 85.4 nM FXa/ 10^8 vp; n=5). The large difference between these viruses in proPL and FXa-generating activity may provide an explanation for why thrombotic lesions are observed with HSV but not for CMV infection (90,111).

3.2.2 Activation of ^{125}I -FX Followed by Electrophoresis

To confirm that exogenous FX was being converted to FXa in the clotting assay, FXa production was followed electrophoretically using radioiodinated FX. As shown in Figure 8, pg 35, a species was produced in the presence of CMV, HSV-1 or HSV-2 and both FVIIa and Ca^{2+} that corresponded exactly to the FXa heavy subunit under reducing conditions. No detectable FXa band was visible when either

FIGURE 7 Virus-dependent generation of FXa clotting activity. CMV, HSV-1 and HSV-2 were incubated with PCPS (300 μ M) in FX deficient plasma for 1 minute at 37°C. FX (200 nM) was added and clot formation was initiated with Ca^{2+} (2 mM). The amount of corresponding FXa activity was determined using a FXa standard curve (inset). (n=5)

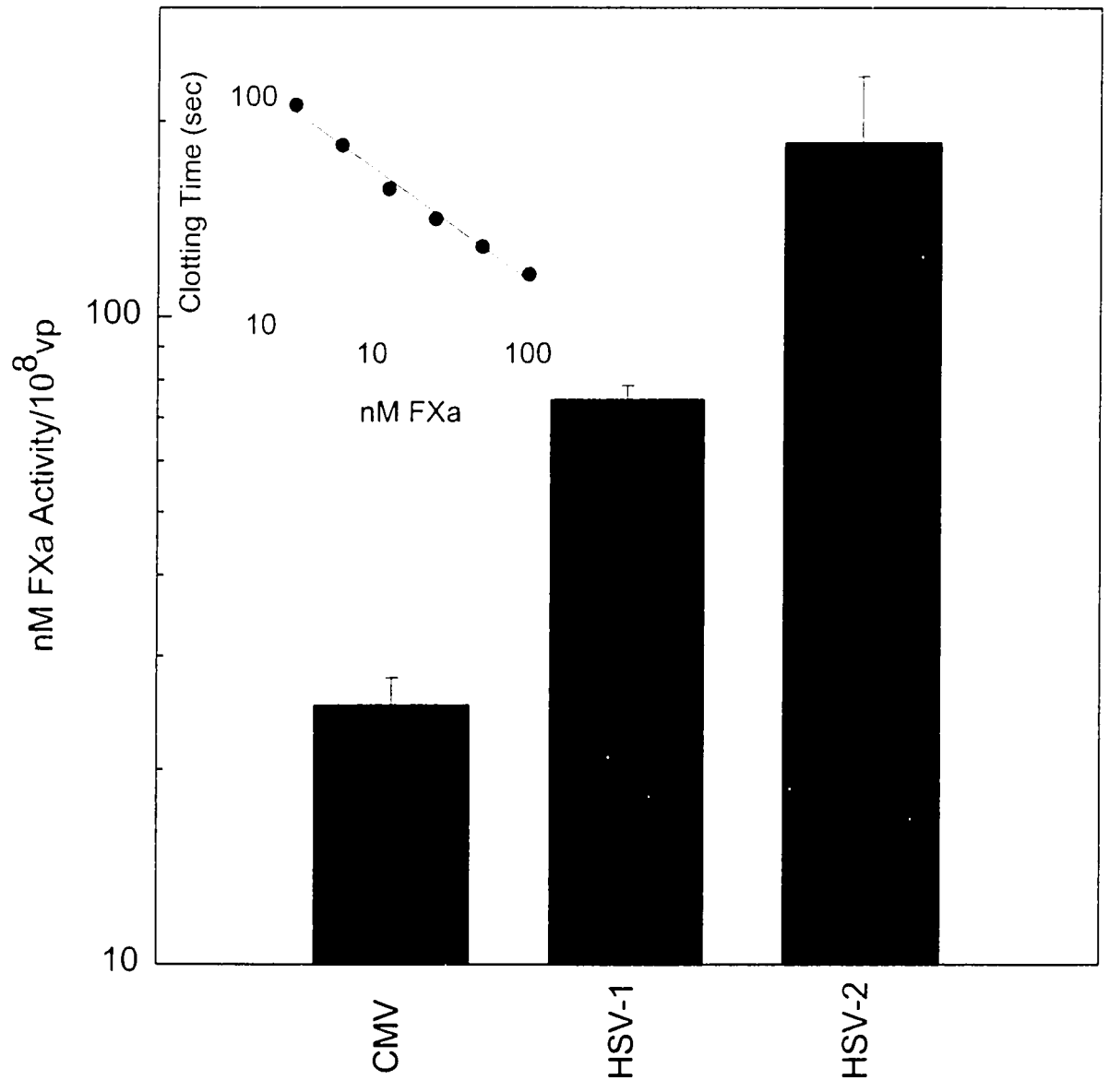
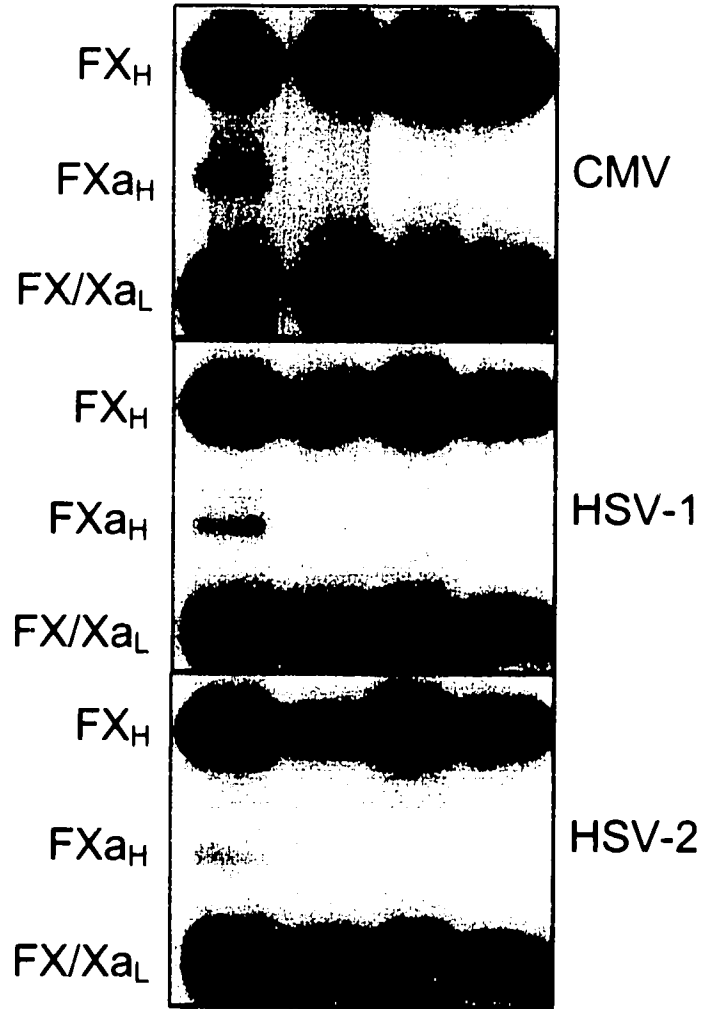


FIGURE 8 Herpesvirus-dependent proteolytic activation of FX. ^{125}I -FX (100 nM) was incubated with CMV, HSV-1, HSV-2 or without virus in the presence of FVIIa (1 nM) and Ca^{2+} . The reaction mixtures were then subjected to reduced SDS-PAGE and the electrophoretic pattern of the ^{125}I under each condition was visualized by autoradiography on Kodak X-OMAT film. The position of the FX heavy (FX_H), FXa heavy (FXa_H) and the FX/Xa light ($\text{FX}/\text{FXa}_\text{L}$) subunits are shown.



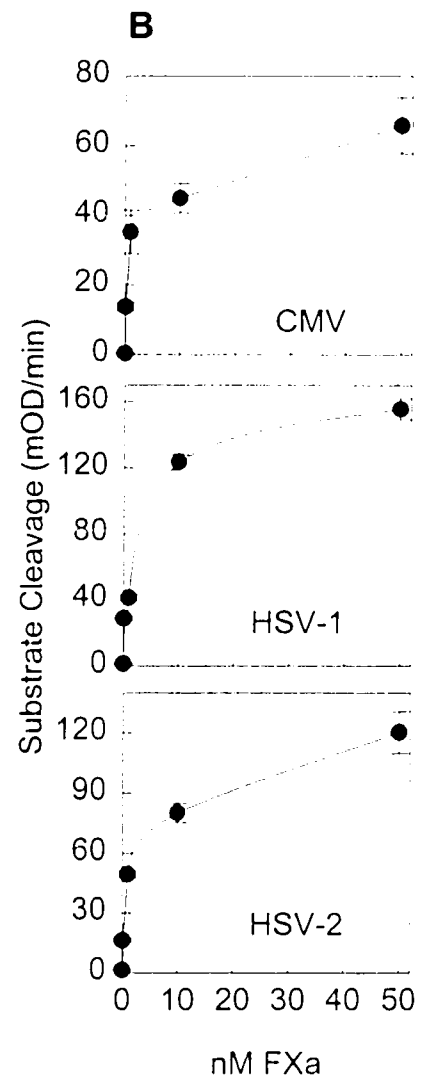
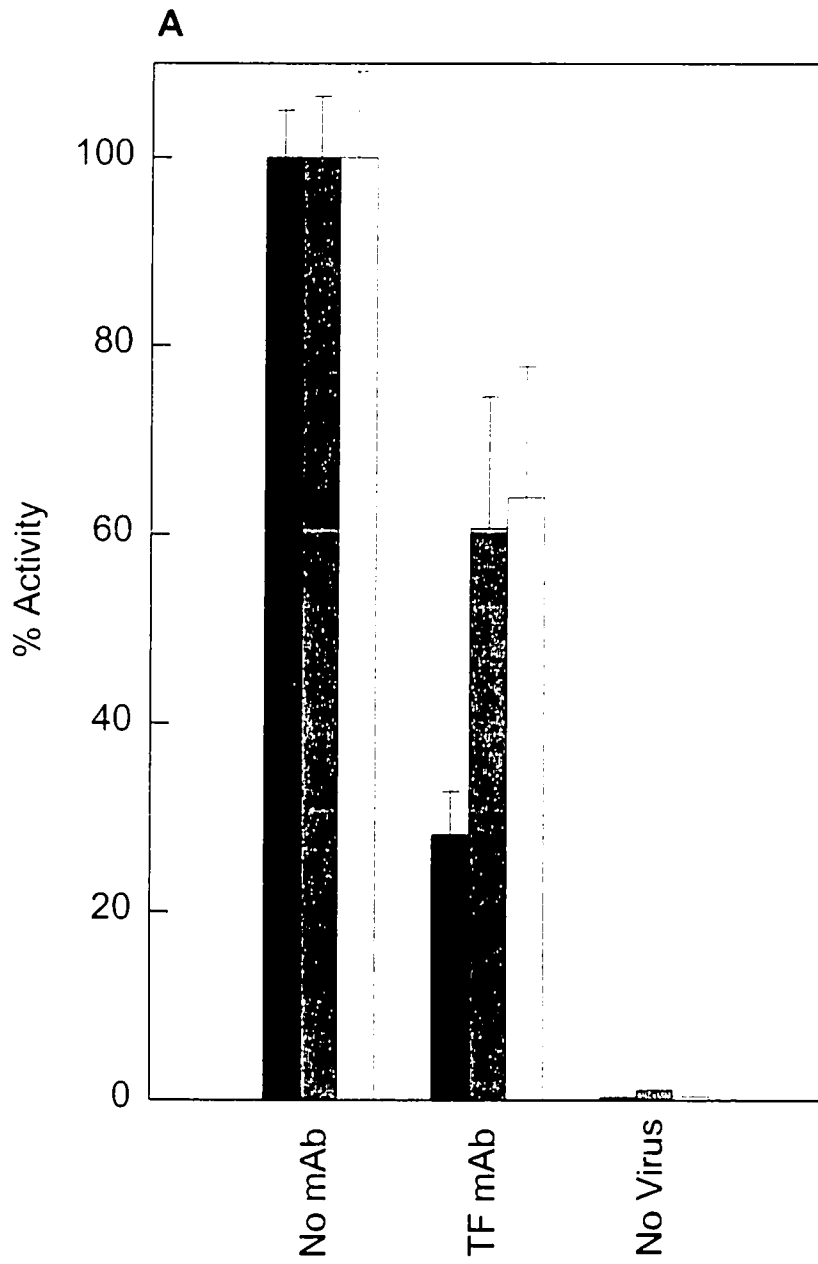
virus, Ca²⁺, Vlla
 Ca²⁺, Vlla
 virus, Vlla
 virus, Ca²⁺

virus, FVIIa or Ca^{2+} was absent. In agreement with the clotting assays, these data show that a TF-like molecule is associated with each virus, which when combined with FVIIa and Ca^{2+} can activate FX to FXa.

3.2.3 Antibody-Mediated Inhibition of TF Activity

The dependence on FVII/VIIIa for FXa generation in the clotting and electrophoretic experiments is consistent with the presence of TF on the virus. In order to further address this possibility, an inhibitory TF-specific mAb was used. Here, purified FX, FVIIa, FVa and prothrombin were sequentially incubated with virus, and thrombin generation was evaluated as a means to identify FX activation. As shown in Figure 9A, pg 37, this experiment revealed that the virus-dependent generation of FXa was inhibited by anti-TF mAb (CMV; 70%, HSV-1; 40%, HSV-2; 35% inhibition). Higher concentrations of antibody did not increase the extent of inhibition (data not shown). The addition of non-immune mouse IgG to the positive control ("no mAb") also had no effect on FXa generation. Additional control experiments showed that this assay was dependent on added Ca^{2+} , FVIIa, FX, FVa and prothrombin. Furthermore, the FX, FVa, prothrombin and chromogenic substrate were added in excess to ensure that Xa generation was the rate limiting step. Figure 9B shows titration curves of activity produced for known amounts of FXa. Conditions were chosen for the immunoinhibition experiment that ensured the

FIGURE 9 Inhibition of virus-dependent FX activation with anti-TF mAb. CMV, HSV-1 and HSV-2 were incubated with either anti-TF (2 μ g), mouse IgG (2 μ g) or antibody for 2 hours at 4°C. Afterward, FX (100 nM), FVIIa (1 nM) and Ca²⁺ (2 mM) were added in the presence of PCPS (300 μ M) for 5 minutes. FVa (3 nM) and prothrombin (1.4 μ M) were then added and the mixture was incubated for a further 25 minutes at room temperature. Thrombin production was measured using S-2238 and activities were normalized to the amount of thrombin generated in the absence of antibody (100%) (A), Black, CMV; Grey, HSV-1; White, HSV-2. (n=9). The amount of FXa produced was determined using a FXa standard curve (B).

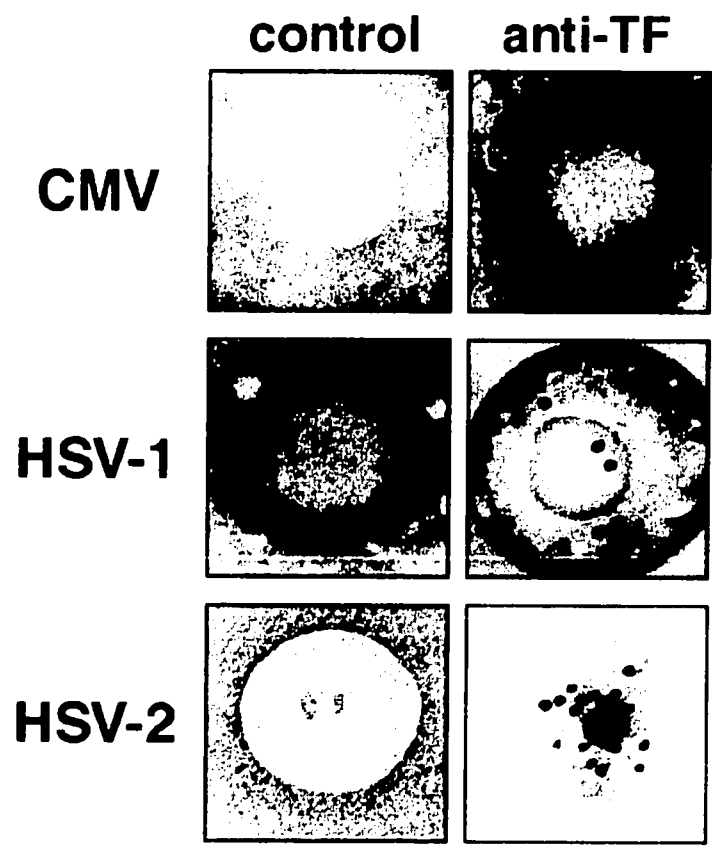


amount of FXa produced did not reach the plateau region shown for each FXa titration curve. The maximum amount of FXa generated in Figure 9A ranged from 1.6 - 2.5 nM for each virus based on calculations of the initial amount of FXa produced in both the electrophoretic and chromogenic assays. Based on the known 1:1 stoichiometry of FXa:FVa (72), the 3nM FVa used was sufficient to bind virtually all of the FXa produced. As well, the known rate of chromogenic substrate cleavage assuming complete conversion of the prothrombin to thrombin (1.4 μ M) was not reached in the system. No endogenous FX/Xa activity was present on any of the three viruses as noted by the inability to detect FXa after incubation of each virus with purified Russell's viper venom FX activator (Haematological Technologies) at 10 μ g/ml for 1 hour at 37°C, 2mM Ca²⁺ (data not shown). These combined functional observations suggest a contribution of TF in the activation of FX by CMV, HSV-1 and HSV-2.

3.2.4 Immunogold Detection of TF Antigen on Herpesviruses

Direct evidence that TF exists on each virus surface was obtained by immunogold electron microscopy (Figure 10, pg 39), in which the electron-dense gold particles identify specific interactions between the anti-TF mAb and each virus type. Approximately 60 - 70% of each of the three virus types were recognized by this antibody. As a negative control, no gold-staining was observed when an

FIGURE 10 Physical demonstration of TF antigen on herpesviruses. CMV, HSV-1 and HSV-2 were evaluated for endogenous TF antigen by immunogold staining using a TF-specific primary mAb under the same conditions as described in FIGURE 3.

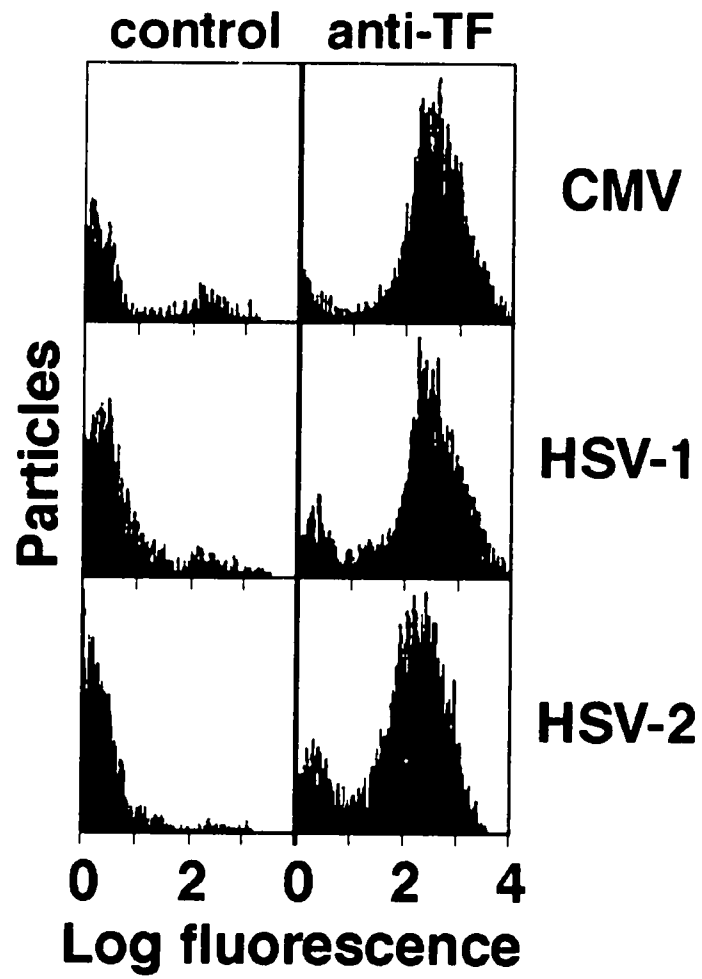


identical amount of anti-actin mAb was substituted for the primary anti-TF mAb. Furthermore, none of the cellular debris in the preparations was shown to be positive for the TF antigen.

3.2.5 Flow Cytometric Detection of TF Antigen on the Herpesvirus Surface

Substantiating evidence that the TF activity is associated with the virus was provided by flow cytometry. As depicted by the histograms in Figure 11, pg 41, 94% of CMV, 88% of HSV-1 and 84% of HSV-2 particles were indeed recognized by the anti-TF mAb. These data demonstrate the presence of TF antigen on virus particles.

FIGURE 11 Demonstration of viral TF by flow cytometry. UV-inactivated viruses were incubated for 1 hour at room temperature with either non-immune mouse IgG (control) or anti-TF mAb (anti-TF). Bound primary antibody was detected by incubation with phycoerythrin-conjugated secondary mAb for 1 hour. The samples were diluted in 1 ml of HBS and the data were immediately acquired using an Epics XL flow cytometer (Coulter) and analyzed using Facsolv-software.



4. DISCUSSION

4.1 THROMBIN PRODUCTION ON HERPESVIRUSES

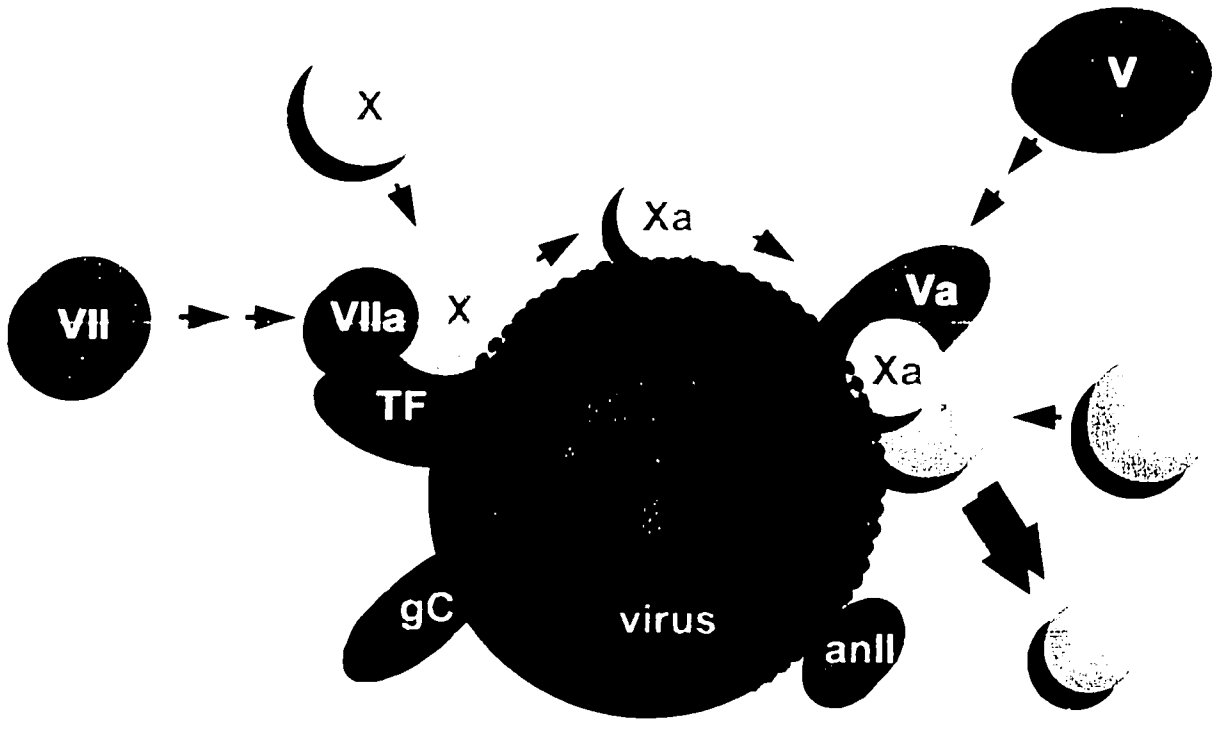
Many types of virus, including herpesviruses, are covered by a membrane that functions in early stages of infection by contributing components necessary for host cell entry (119,120) and for evasion of the immune system (43). This "envelope" contains proteins that are encoded by both the virus and host genomes, with a phospholipid component derived solely from the host cell. Under normal resting conditions, cells actively maintain an asymmetric phospholipid distribution with proPL located on the inner side of the lipid bilayer (20,151). Thus, membrane sidedness acts as an important hemostatic barrier that limits the exposure of proPL to sites of vascular damage, where it is "flipped" to the surface. In a previous study (114), we demonstrated that the CMV envelope has proPL on its surface and consequently circumvents an important cellular hemostatic control. Through the use of clotting and chromogenic assays, flow cytometry and electron microscopy, the current study extends this finding to include both HSV-1 and HSV-2 as having accessible proPL.

In order to initiate thrombin production, proPL and a mechanism to convert FX to FXa must become available to the circulating plasma coagulation proteins. Under normal physiological conditions, the first FXa molecules are made by the TF/FVIIa tenase (85). Both HSV-1 and CMV have been demonstrated to enhance

TF expression on host endothelial cells (138,139). Similarly, experiments using UV inactivated viruses revealed an increase in TF activity on the cultured cells we used to propagate viruses (data not shown). Therefore, it was speculated that TF produced in response to infection may be routed to the virus particle when the envelope is formed. It was found that all three viruses can participate in the activation of FX, as concluded from clotting, electrophoretic and chromogenic data. For each, the activation of FX was dependent on Ca^{2+} and FVIIa, which suggested the presence of a TF-like constituent on the virus surface. Using a mAb raised to TF, it was demonstrated by flow cytometry and electron microscopy that TF antigen is present on virions. Furthermore, a different TF mAb inhibited the virus-dependent generation of FXa.

As summarized in Figure 12, pg 44, the data presented cumulatively demonstrate that CMV, HSV-1 and HSV-2 each possess the molecular machinery necessary to initiate thrombin generation on their envelope surface. This activity involves endogenous proPL (light colored polar head groups in Figure 12), to facilitate coagulation enzyme-cofactor-substrate complex assembly, and a species functionally and antigenically identical to TF, which participates in FX activation. Since neither the CMV nor HSV genome encode a TF homologue, it must be acquired along with proPL from the host cell during virus envelope formation. Therefore, the amount of TF activity found on the viruses may at least in part be due

FIGURE 12 Model for thrombogenic mechanisms identified on herpesviruses. CMV, HSV-1 and HSV-2 each possess the molecular machinery to initiate thrombin (IIa) generation on their envelope surface. This involves endogenous proPL (light colored head groups) and tissue factor (TF) activity to allow tenase (TF, factor VIIa (FVIIa)) and prothrombinase (factor Va (FVa), FXa) complex assembly and respective activation of FX to FXa and prothrombin to thrombin. Each step required Ca^{2+} .



to the amount found associated with the different host cells. Each of the three viruses also had the ability to activate FVII from plasma in the clotting assays and in the purified system. When FVII was substituted for FVIIa in the purified system, the activity generated was approximately 50% of that produced when FVIIa was used, indicating that not all of the FVII was activated on the virus under these conditions. The production of FVIIa in either of these instances may have resulted from autoactivation by the FVII/TF complex or another protein on the virus surface. Interestingly, the viruses were also capable of activating FV in the clotting assays. This may be due to the initial generation of FXa which acts in an amplification mechanism to generate FVa to be used in the prothrombinase complex. Alternatively, a protein on the virus, either host or virus encoded, may cause the generation of FVa.

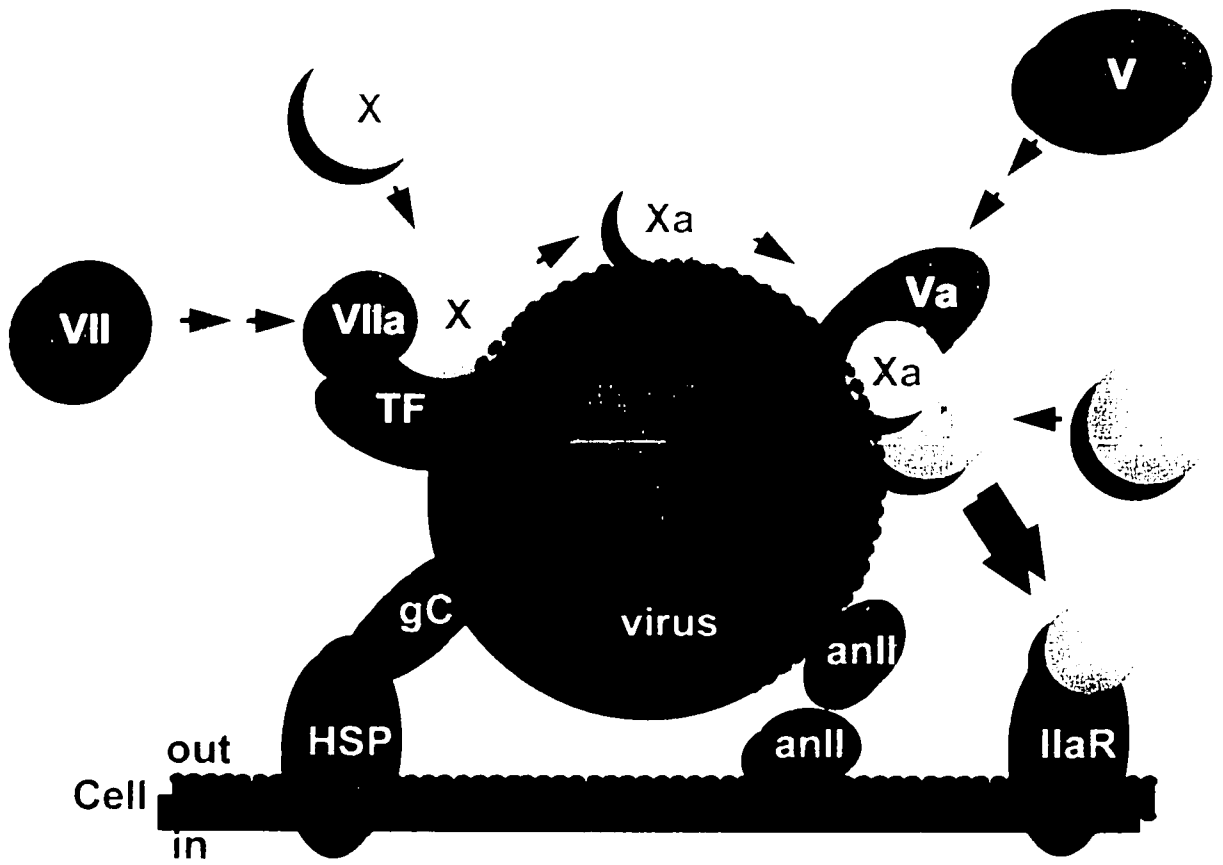
Other components on the virus may contribute to thrombin production. One example is the HSV-1-encoded gC. This glycoprotein has been shown to accelerate FX activation when expressed on the surface of infected endothelial cells (9). The purified virus is known to have gC on its surface (43), but whether or not it can function like the cellular form within a tenase complex has not been determined. This would provide a possible explanation for the incomplete inhibition of FXa generation observed by using the anti-TF mAb. Another explanation is that the mAb, which is raised to human tissue factor, may not inhibit completely TF acquired

from the monkey-derived host cells (Vero cells), used to propagate HSV-1 and HSV-2. Since FXa generation by virus alone was not observed in the electrophoresis experiment suggests that the activity not inhibited by the TF antibody may be dependent on FVIIa.

The incomplete inhibition of TF activity is the first evidence that the virus-encoded gC protein may be involved in FXa generation directly on the virus. This is interesting since it implies that there is an evolutionary advantage for the virus to generate thrombin before cell entry. Since HSVs have gC and CMV does not, these studies may explain why HSV was observed to have at least two orders of magnitude higher thrombin generating activity per particle compared to CMV (127). This finding may also explain why severe HSV infection leads to fibrin deposition in microvasculature (77,90) and disseminated intravascular coagulation (111) in humans, but thrombotic complications due to CMV are rare.

A hypothetical extension of the model in Figure 12 is shown in Figure 13, pg 47 that includes the cell modulatory effects of thrombin. It is based on the observation that CMV, HSV-1 and HSV-2 can initiate thrombin production on their surface. The amount of thrombin that can be produced on a single blood-borne virus particle is unlikely sufficient to overcome the anticoagulant threshold of plasma. However, when CMV, HSV-1 and HSV-2 bind near thrombin receptors on a host cell surface or when a large local concentration of virus is released upon

FIGURE 13 Cell modulation by herpesvirus-produced thrombin. The thrombin (IIa) that is produced by the sequential activation of coagulation factors on the virus may be a triggering event. When the virus binds near thrombin receptors (IIaR) on a host cell surface (via initial interactions with cell proteoglycans, HSP, by gC on the virus) the thrombin produced on the virus surface can result in thrombin receptor perturbation. This may signal cellular events that lead to enhanced infection. One such event may be the exposure of annexin II (AnII), a putative viral receptor.



cell lysis, the probability of thrombin inhibition is decreased. Thus, thrombin production on the virus is seen primarily as a cell triggering event, which may have two roles in pathology. First, thrombin receptor perturbation may signal cellular events that lead to enhanced infection. Second, herpesviruses establish latent infection, which means that the host is exposed to numerous recurrent, subclinical infections. The resulting procoagulant and proinflammatory effects of small amounts of localized thrombin production would be cumulative and predispose to vascular disease (48,94,138). The induction of cellular thrombogenic activity by viral thrombin may furthermore propagate the effect and establish localized areas of vasculature that are especially susceptible to infection.

4.2 POSSIBLE EFFECTS OF HERPESVIRUS-PRODUCED THROMBIN

4.2.1 Infection

The ability of viruses to convert resting cells from an anticoagulant to a procoagulant state is not unique to herpesviruses. Measles virus, murine hepatitis virus and avian hemangioma retrovirus also cause host cells to become thrombogenic (44,86,116). It is therefore possible that virus infection in general may cause certain procoagulant effects. Perhaps more importantly, the ability of thrombin to function as a potent cell modulator may be exploited by the viruses as a way to signal host cells during infection. Both herpesviruses and thrombin have

been shown to trigger similar intra- and extracellular events, many of which can be attributed to G protein-coupled receptor perturbation (142,149). The current data suggest that the production of thrombin on virus attached to the host cell surface may therefore be an initiating event in the infection process and vascular pathology mediated by herpesviruses. There are several points along the pathway leading to CMV and HSV infection that may be enhanced by thrombin.

4.2.1.1 Attachment to the host cell surface

The attachment of CMV and HSV to host cells is a sequential process that is dependent on initial interactions with cell surface heparan sulfate proteoglycan (HSP) (64). Currently there have been several viral glycoproteins implicated in this process. These include the CMV encoded glycoprotein C-II (gCII) (64) and glycoprotein B (gB) (28), while gC (55) and gB (54) have been identified for HSV. This interaction, which is inhibited by soluble heparin, prepares the cell for a second attachment step.

Experiments intended to identify the second site of attachment for CMV on the host cell implicated AnII as a CMV-binding protein (80,146). The discovery of a cell surface component with electrophoretic properties identical to AnII that correlated with viral tropism (107) and a report that an antiserum raised against AnII inhibited infection (145) provide indirect support for the receptor role of AnII. There

has also been direct evidence that purified AnII can independently function as a CMV receptor and enhance viral plaque formation (115). Interestingly, our preliminary results showed that cell stimulation with thrombin caused an increase in the amount of AnII detected on the surface. The virus may therefore induce the expression of its own receptor by producing thrombin as a trigger.

4.2.1.2 Penetration of the host cell membrane

After the initial contacts with the host cell have been made, pH-independent fusion of the CMV or HSV envelope with the cell membrane occurs. For CMV, this is believed to involve the virus-encoded glycoprotein H (gH) and gB (65). An anti-idiotypic antibody that mimics the cell binding site on gH showed that a 92.5 kDa host cell cognate receptor was involved in virus penetration (17) and also functions in phosphorylation by protein-tyrosine kinases (66). PKC activity, which is increased by thrombin, has also been correlated to virus infection (125). A number of HSV glycoproteins have been identified that are important for penetration of the host cell membrane and include gB, gD, and the gH/glycoprotein L complex (25,78,121,123). A potential link between HSV-1 penetration and thrombin stimulation is implied by observations, like those for CMV, that PKC inhibitors attenuate the entry of virus into fibroblasts (29).

4.2.1.3 Transcription of the First Virus Genes

The transcription of CMV- and HSV-encoded genes is sequential with those designated "immediate early" (IE) being first expressed. These depend heavily on host transcription factors and do not require the prior expression of viral genes. These genes have the ability to function as transcription factors and are essential for the expression of later viral genes. Host transcription factors in CMV infection are important in the regulation of the major IE promoter (MIEP). There are several host cell proteins known to activate the MIEP. The functioning of NF- κ B in this process is considered to be fundamental (96) and is important within the context of thrombin-mediated receptor activation. Prior to stimulation, NF- κ B is stored in a cytoplasmic pool primarily bound to the inhibitory protein, I- κ B. Several reports have demonstrated that CMV treatment of host cells enhances NF- κ B activity within 2 hours (21,69). This effect does not require protein synthesis and occurs through mobilization of the cytoplasmic store to initiate CMV IE gene transcription (148). Thus, an additional involvement of thrombin in CMV infection may be at this point of regulation because thrombin is known to rapidly enhance NF- κ B activity (123). In fact, for some of the reported experiments, thrombin production on the virus surface could have been a contributing factor because the necessary exogenous coagulation proteins (FX, FVII, FV, prothrombin) are provided within culture media containing serum.

The expression of the HSV-1 IE genes also requires the contribution of host cellular transcription factors. There is a strong dependence on a virus tegument phosphoprotein, termed VP-16, present in the upstream promoters of the HSV IE genes (97,120). VP-16 phosphorylation has been reported as essential for subsequent complex formation. This event may be affected by thrombin receptor perturbation which mediates activation of various kinases.

4.2.2 Vascular Disease

Thrombin is the biological effector of the coagulation pathway and functions as a cell modulator (34,83). Responses to thrombin include stimulation of smooth muscle cell proliferation, recruitment of monocytes and macrophages, and the development of a procoagulant phenotype, all of which may lead to atherosclerosis. Many of these same cellular changes are caused by CMV and HSV infection and may be initiated by the thrombin produced on the virus surface. The involvement of thrombin in these changes is supported in the literature showing that hirudin, a specific thrombin inhibitor, reduced monocyte adhesion to infected vascular endothelial cells (38) and similarly, adhesion was inhibited by depleting the inoculation media of prothrombin (38). The ability of CMV and HSV to promote the generation of thrombin as a cell stimulant may be the earliest event linking subclinical CMV and HSV infections to atherogenesis (92,94,138).

4.3 CONNECTION BETWEEN OTHER VIRUSES AND proPL

While the three viruses that were evaluated here are all from the herpesvirus family, it is conceivable that accessible proPL is a ubiquitous characteristic of enveloped viruses. It has been shown that human immunodeficiency virus type 1 and type 2, vesicular stomatitis virus, extracellular autographa californica nuclear polyhedrosis virus and HSV all have a higher proportion of PS in their envelope than the host cell membrane (5,22,82,137). There is further evidence in the literature that identifies a role for PS and PS-binding proteins in hepatitis B, influenza A and B, hemorrhagic septicemia virus, sendai virus, vesicular stomatitis virus, rubella virus, sindbus virus, vaccinia virus and CMV infection (27,37,74,82,86,105,108). Therefore, the observation that CMV, HSV-1 and HSV-2 can assemble prothrombinase on their surface may be a consequence of a distinct function for proPL in the infection process. In this regard, the proPL can function as an anchor for the proPL-binding protein AnII, which is a CMV receptor (115,145,146). Therefore, the current findings may define an involvement of thrombin in general enveloped virus infection and suggest that a hypercoagulable state may predispose to infection.

4.4 FUTURE STUDIES

4.4.1 Determine if Other Thrombogenic Mechanisms Exist on Herpesviruses

The finding that a mAb raised against TF only partially inhibited the generation of FXa on CMV, HSV-1 and HSV-2, suggests that other mechanisms may exist. This is especially true for the HSVs, which are maximally inhibited by 40% compared to 80% for CMV (127). The possibility of an additional tenase on HSV-1 is supported by an earlier report showing that HSV-1 gC expressed on the surface of an infected cell can participate in FX activation. Further inhibition of FXa generation by HSV-1 using a combination of TF and gC antibodies will be attempted as well as the use of HSV-1 gC mutant strains to determine the function of gC in a tenase complex. The fact that HSV-1 encoded gC and possibly constituents of CMV and HSV-2, have evolved to activate FX suggests a role for thrombin in virus infection.

4.4.2 Determine the Role of Thrombin Produced on the Virus

Preliminary data has shown that CMV infection of host cells is enhanced by pretreating the cells with thrombin. Therefore, the ability of thrombin generated at the virus surface to trigger cellular events will be investigated. The parameters that will be initially focused upon will include the effects on overall infection and virus receptor expression. In order to distinguish effects induced by: a) thrombin

produced on the virus; b) thrombin produced on the cell; and c) direct virus-cell interactions, the use of purified coagulation factors and a series of preincubation/washing steps will control where and how thrombin is being produced. In this way, it is anticipated that the reason herpesviruses have evolved to generate thrombin will begin to be understood.

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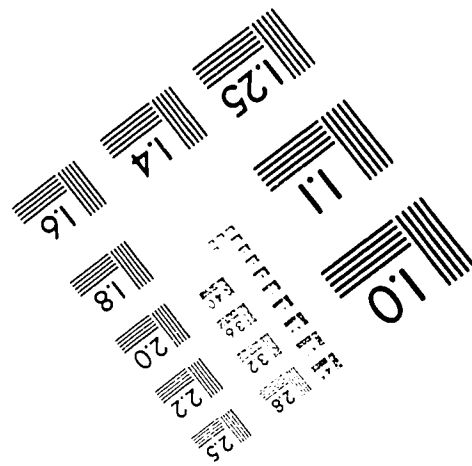
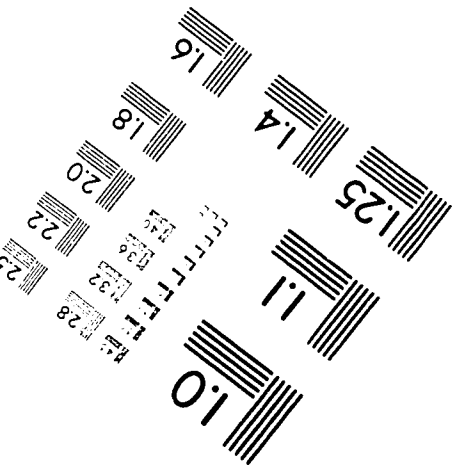
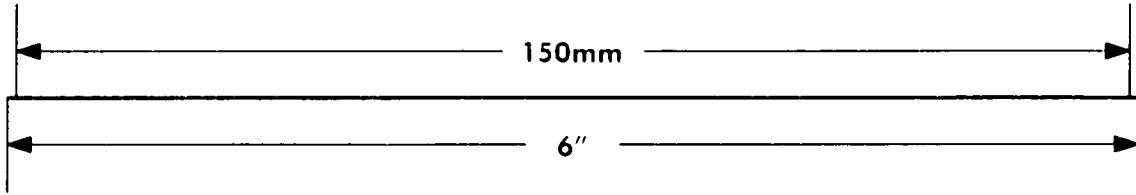
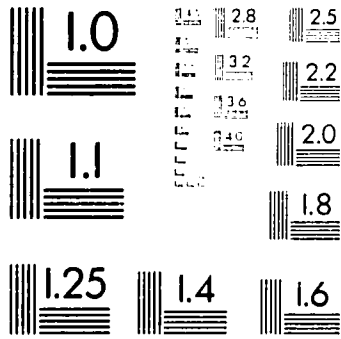
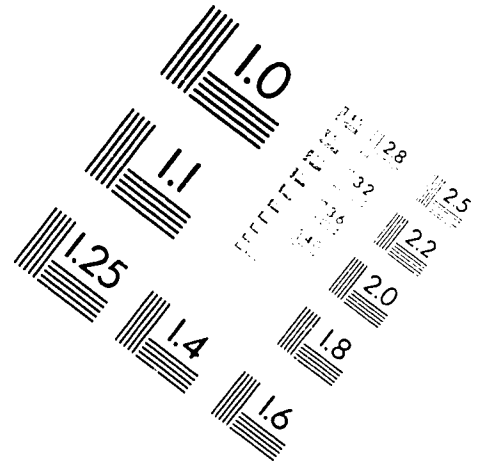
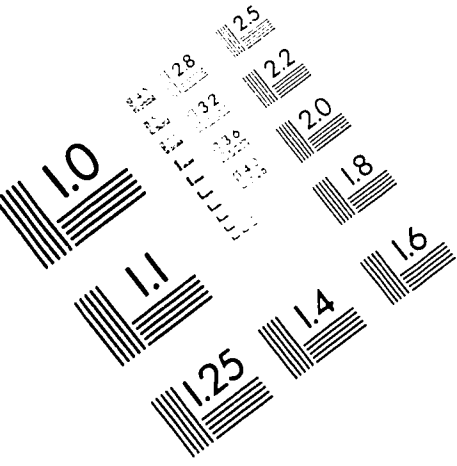
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