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Cytomegalovirus induced PMN adherence in relation to an ELAM-1 antigen present on infected endothelial cell monolayers

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SUMMARY

In human umbilical vein endothelial cells infected with cytomegalovirus (CMV), an activation antigen recognized by monoclonal antibody (mAb) ENA1 appeared. mAb ENA1 reacts with an inducible endothelial surface antigen which has characteristics similar to those of ELAM-1. Incubation with anti-IL-1 partly inhibited this appearance and, parallel to this, the virus-induced polymorphonuclear cell (PMN) adhesion was decreased. In addition, the adhesion of PMN to virus-infected endothelial cells could be reduced by $F(ab)_2$ fragments of mAb ENA1 to almost control level. The results obtained after incubation of PMN with mAb IB4 (against CD18) suggest that the adhesion of PMN to uninfected endothelial cells is CD18 glycoprotein dependent, and virus infection up-regulates this glycoprotein-dependent mechanism. These results indicate that the virus-induced PMN adhesion is regulated by the following mechanism: virus infection of endothelial cells induces IL-1 production, and the autocrine IL-1 causes the expression of ELAM-1 on the surface of endothelial cells. In turn this activation antigen ELAM-1 binds with its putative ligand present on the PMN membrane. The virus-induced PMN adhesion occurs also through a CD18 glycoprotein-dependent mechanism.

INTRODUCTION

Endothelial damage is considered to be an important early event in the pathogenesis of atherosclerosis.[1] It has been proposed that endothelial damage by herpes viruses may play a role in the etiology of atherosclerosis. The association of herpes virus infection and atherosclerosis is based on several observations. Marek's disease, caused by an avian herpes virus, induces atherosclerotic lesions, which closely resemble human atherosclerotic lesions.[2] In man, herpes virus particles have been observed by electron microscope in the proximal aorta of atherosclerotic patients,[3] and cytomegalovirus (CMV) antigens[4] and CMV nucleic acids[5] have been detected within atherosclerotic plaques. The mechanisms by which herpes virus infection does cause endothelial damage are not clear, and several possibilities have been put forward. Infection and reactivation of latent infection might lead to actual endothelial lysis.[6] Or, as hypothesized by Visser, inflammatory cells are attracted to and marginate on virus-infected endothelium and initiate the development of atherosclerosis.[7] Regarding this last mechanism, others have shown that herpes virus infection enhances granulocyte adherence to endothelial cells.[8-10] However, the cellular mechanisms by which virus induces this effect are still not defined. It is known that virus infection induces changes in the endothelial plasma membrane, like the appearance of Fc and C3b endothelial receptors.[11,12] Further virus infection results in an abrogation of prostacyclin synthesis and in an increased thrombin generation,[7] through which endothelial cell properties shift from anti-coagulant to procoagulant. Van Dam-Mieras[13] observed that CMV infection of endothelial cells resulted in an increased procoagulant activity. Another possibility, by which virus induces an enhanced granulocyte adhesion, is the secretion of soluble factors by endothelium after virus infection,[9] such as prostaglandins,[14] platelet-activating factor (PAF)[15] and interleukin-1 (IL-1).[16,17] From this last mediator it is known that it stimulates the endothelial plasma membrane, resulting in an avid adhesion of neutrophils, monocytes[18] and lymphocytes.[19] By others it is demonstrated that ICAM-1, ICAM-2,[20] GMP-140[21] and ELAM-1[22,23] are involved in this leucocyte adhesion. Recently, another cytokine-inducible endothelial cell receptor for neutrophils was described.[24] This antigen, recognized by monoclonal antibody (mAb) ENA1, is rapidly redistributed to the plasma membrane during activation and is structurally related to ELAM-1.[25]

This study concerns the hypothesis that virus infection leads to activation of endothelial cells, which results in interleukin-1

Abbreviations: CMV, cytomegalovirus; FCS, foetal calf serum; HEF, human embryonal fibroblast; IL-1, Interleukin-1; mAb, monoclonal antibody; PMN, polymorphonuclear leucocyte.

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(IL-1) secretion. This IL-1 stimulates the endothelial cells causing a changed antigen membrane expression and an increased leucocyte adhesion. The role of the activation antigen ELAM-1 is investigated within this process. For this purpose, endothelial cell monolayers were infected with CMV. At different hours post infection (p.i.), viral and surface antigen expressions were determined and polymorphonuclear (PMN) cell adhesion was measured.

MATERIALS AND METHODS

Reagents

The culture media were purchased from Gibco Biocult Co (Paisley, Renfrewshire, U.K.). Foetal calf serum and newborn calf serum were obtained from Boehringer (Mannheim, Germany) and Gibco Biocult Co., respectively. Heparin was obtained from Serva (Heidelberg, Germany). Cell culture trays were obtained from Costar (Cambridge, MA). Nonidet P40 (NP40) for fixation of cell monolayers was obtained from Sigma (St Louis, MO). Fibronectin was acquired from the central Laboratory of The Dutch Red Cross Transfusion Service (CLB), Amsterdam, The Netherlands. FITC-conjugated rabbit $F(ab)_2$ anti-mouse serum was obtained from Dako (Amstelstad Zwanenburg, The Netherlands). Peroxidase-conjugated goat anti-mouse gammaglobulin was obtained from Jackson Immuno Research Laboratories (West Grove, PA). Glutaraldehyde for fixation of the cell monolayer and paraformaldehyde were obtained from Merck (Darmstadt, Germany). A polyclonal rabbit anti-natural human IL-1, which inhibits the biological activity of IL-1, was kindly provided by Dr J. van Damme, Rega Institute of Medical Microbiology, University of Leuven, Belgium. Monoclonal antibody IB4, directed against the common beta chain (CD18) of LeuCAM, was a generous gift from Professor M. Daha, University Hospital Leiden, The Netherlands. Monoclonal antibody ENA1, reactive with an inducible endothelial surface antigen with characteristics similar to those of ELAM-1,[24] was a generous gift from Dr W. A. Buurman, University of Limburg, The Netherlands. This monoclonal reacts specifically with endothelial cells activated with tumour necrosis factor (TNF), IL-1, lipopolysaccharides (LPS) or phorbol esters and is not reactive with other cell types.

Cells

Human endothelial cell monolayers were established from cells, obtained by trypsin treatment of umbilical cord veins as described by Bruggeman *et al.*[26] The cells were grown in culture medium consisting of 50% medium 199 (M199) and 50% RPMI-1640 supplemented with heat-inactivated 20% foetal calf serum (FCS), heparin (10 U/ml), 0·2% endothelial growth factor (EGF)[27] and antibiotics. The endothelial cell monolayers were used after five to eight doublings and were nearly confluent at the time of viral infection. The monolayers were grown in 90-mm plastic Petri dishes and for the adhesion assay in 96-well tissue culture cluster plates. For antigen detection studies the monolayers were grown also in 96-well tissue culture cluster plates containing 10^4 cells/well.

Human embryonal fibroblasts (HEF) were cultured in Eagle's minimal essential medium (MEM) supplemented with heat-inactivated (at 56° for 30 min) 10% newborn calfserum (NCS) and antibiotics. Confluent HEF monolayers were used for the preparation of the virus pool.

Human polymorphonuclear leucocytes (PMN) were isolated from buffy coats, obtained after cytophoresis of blood of healthy donors, using the method described previously.[10] The viability of isolated cells was determined by counting the percentage of cells that excluded trypan blue and was consistently found to be > 95%. The purity of the harvested PMN was assessed by morphology as well as by chloro-acetate esterase staining[28] and > 90% of the cells were judged to be PMN. The isolated PMN were stored on ice in Ca/Mg-free Hanks' balanced salt solution (HBSS) supplemented with heat-inactivated 1% FCS. Before use in the adhesion assay experiments cells were resuspended in RPMI-1640 supplemented with 1% FCS.

Viral techniques

Cytomegalovirus (CMV) (Kerr strain) was grown in HEF monolayers and virus stocks with titres between 5 and 10×10^7 PFU/ml were prepared. The infectivity of the CMV pools was determined by plaque assay in HEF, as described for rat CMV.[29]

Unless indicated otherwise, endothelial cell monolayers were infected with CMV at a multiplicity of infection (MOI) of 30, using the technique described previously,[10] and were used in the adhesion assay at 24 hr post-infection (p.i.). Virus antigen expression was determined, at different hours p.i., by means of an enzyme-linked immunosorbent assay (ELISA) or by an indirect immunofluorescence (IF) assay using CMV monoclonal antibodies (mAb). The two mAb H219 and H222, directed against CMV antigens, were prepared according to standard procedures.[30] mAb H222 gives a positive staining in the nucleus, while mAb H219 is directed against cytoplasmic CMV antigens. No cross-reactivity with other herpes viruses could be detected.

For IF, virus-infected endothelial cell monolayers were fixed with 3% paraformaldehyde at room temperature for 10 min, then incubated with mAb H222 (dilution 1/50) for 45 min at 37°, washed in phosphate-buffered saline and further incubated with FITC-conjugated rabbit anti-mouse gammaglobulin (dilution 1/25) for 30 min. After washing, treated and non-treated endothelial cell monolayers were observed simultaneously in a Zeiss fluorescent microscope equipped with the appropriate barrier filters. Data are presented as percentage of cells which gave a positive staining/microscopic area.

For the detection of viral antigens in ELISA, endothelial cell monolayers were fixed with 3% paraformaldehyde supplemented with 0·1% Triton X-100. After fixation the viral antigen expression was determined using mAb H219 and goat anti-mouse peroxidase; O-phenylenediamine dihydrochloride (OPD) (Sigma) was used as substrate. The plates were read on a Microtitre ELISA reader at 492 nm. The data are reported as the mean absorbance units from six- to eightfold determinations after subtraction of the background. The SD never exceeded 5% of the mean value. Control monolayers were incubated with medium harvested from uninfected monolayers and were processed in a similar manner.

Induction and detection of an activation antigen ELAM-1 in CMV-infected endothelial cell monolayers

The expression of the ELAM-1 antigen was determined by ELISA in CMV-infected monolayers at different times p.i. In control experiments endothelial cell monolayers treated with IL-1 were used. For the detection of ELAM-1 by IF, mono-

layers were fixed with 3% paraformaldehyde and observed in a Zeiss fluorescent microscope. The data are presented as percentage of cells which gave a positive staining/microscopic area.

Detection of IL-1 activity in supernatants derived from CMV infected endothelial cells

To detect whether infected endothelial cell monolayers excreted IL-1, supernatants from infected and non-infected endothelial cell monolayers were collected, at 24 hr, 48 hr and 72 hr p.i. Until used, these supernatants were stored at −70°. IL-1 activity was measured by using the bioassay based on sublines of the D10G4.1 (D10) murine T-cell line, as described by Hopkins & Humphreys.[31]

Inhibition of the ELAM-1 expression

To inhibit the virus-induced ELAM-1 expression, virus infected- and non-infected monolayers were incubated with anti-IL-1 during and after infection. At 12 hr p.i., the ELAM-1 expression was determined in ELISA. Controls consisted of infected and non-infected endothelial cell monolayers incubated without anti-IL-1. The data reported are mean values from eightfold determinations and the SD never exceeded 5% of the mean value.

Adhesion assay

Leucocyte adhesion to cultured endothelial cells was evaluated by a radiometric assay in which the PMN were labelled with ^{51}Cr (100 μCi/10^7 cells, specific activity 5 mCi/mg; Amersham, Amersham, Bucks, U.K.) for 45 min at 37°. The labelling reaction was stopped by the addition of cold buffer. Excess ^{51}Cr was removed by rinsing the labelled cells in excess buffer before addition to the monolayer. Endothelial cell monolayers were incubated with PMN for 15 min at 37°. After removing the supernatant, the monolayers were rinsed five times with medium RPMI-1640 supplemented with 20% FCS and successively lysed with 1·25% EDTA trypsin. The cell fractions were counted on a gamma counter. The mean of eight replicate samples is presented and the data are expressed as percentage of attached c.p.m. to total c.p.m.

To inhibit autocrine release of IL-1, anti-IL-1 was added to the cell monolayer during the experimental period. At 24 hr p.i. the PMN adhesion was measured. Controls consisted of cell monolayers without anti-IL-1 incubation.

In order to block the activation antigen expressed on virus-infected cell monolayers, endothelial cells were incubated for 30 min with $F(ab)_2$ fragments of mAb ENA1 [$F(ab)_2$ mAb ENA1] prior to adhesion evaluation. At 24 hr p.i. the leucocyte adhesion was determined as described above. As controls, infected and non-infected monolayers without $F(ab)_2$ mAb ENA1 incubation were used.

To evaluate whether the PMN adhesion to endothelial cell monolayers acts through the common beta chain (CD18) of LeuCAM, PMN suspensions were incubated with or without the monoclonal antibody against CD18 (mAb IB4) during 15 min, before addition to the infected or non-infected monolayers.

Statistical analysis

To test the effect of virus infection on the PMN adhesion the Wilcoxon test was used. If the P value was $\leqslant 0{\cdot}05$, the difference between the values was determined as being significant.

RESULTS

ELAM-1 and viral antigen expression

Endothelial cells were examined by ELISA for the expression of the antigen, reactive with mAb ENA1, at 0, 3, 6, 12, 15, 20, 24, 28 and 44 hr p.i. Viral antigen expression was measured in the same experiment. Figure 1 shows the results of a representative experiment. The expression of the antigen reactive with mAb ENA1 was parallel to the viral antigen expression. Both were optimal after 12 hr; thereafter the expressions declined. At 20 hr p.i. the expressions reached baseline values.

Control experiments, using IL-1, a known inducer of ELAM-1,[24] showed that the activation antigen expression was maximal after an incubation time of 6 hr; thereafter the expression declined (Fig. 2).

In additional experiments the ELAM-1 expression was determined by IF in virus- or IL-1-treated endothelial cell monolayers counting the number of mAb ENA1-positive cells. A maximal antigen expression was detected at 12 hr p.i. and after 6 hr treatment with IL-1 (data not shown).

To assess whether the mAb ENA 1-positive cells were the CMV-infected endothelial cells, a double IF was performed. Infected and non-infected endothelial cell monolayers were

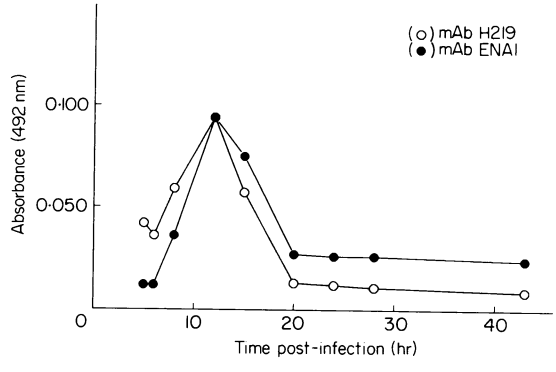

Figure 1. Expression of virus antigen and activation antigen ELAM-1 on CMV-infected endothelial cell monolayers at different hours post-infection. The antigen expression was measured in ELISA and the values, expressed in absorbance units, are the mean of six- to eightfold determinations, as indicated in the Materials and Methods.

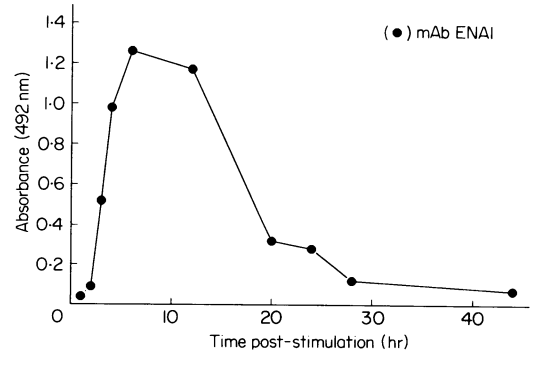

Figure 2. Expression of activation antigen ELAM-1 on IL-1-treated endothelial cell monolayers after different hours of stimulation. The ELAM-1 expression was measured in ELISA, and the values are expressed in mean absorbance units from six- to eightfold determinations, as indicated in Materials and Methods.

incubated with a mixture of mAb H222 (to detect viral antigens) and mAb ENA1 (to detect ELAM-1) and examined at 6, 12 and 24 hr p.i. (Table 1). The infected endothelial cell monolayers expressed both mAb ENA1-positive cells, giving a weak fluorescent signal on the cell-surface membrane, and CMV-positive cells, giving a strong immunofluorescence in the nucleus. However, the endothelial cells which were mAb ENA1 positive did not give a positive fluorescence with mAb H222. Furthermore, it was not possible to discriminate CMV- plus ENA1-positive cells from only CMV-positive cells.

IL-1 production and effect of anti-IL-1

The production of IL-1 after CMV infection was measured at 24, 48 and 72 hr p.i. No IL-1 activity was measured in the supernatant during the experimental period. In a simultaneous experiment we studied, by incubating with anti-IL-1, if the expression of the antigen reactive with mAb ENA1 was mediated by autocrine IL-1 release. After CMV infection an increased antigen expression, 160% of the control expression was detected in ELISA. Incubation with anti-IL-1 significantly reduced this increase of ELAM-1 expression by 75%.

Adhesion experiments

Parallel to the antigen detection experiment the adhesion of ^{51}Cr-labelled PMN to CMV infected and non-infected endothelial cells was measured. In addition, it was examined if the activation antigen ELAM-1 and IL-1 production were involved in the virus-induced PMN adhesion by pretreatment of endothelial cells with anti-IL-1 or with $F(ab)_2$ fragments of mAb ENA1.

Figure 3 demonstrates that the PMN adhesion to CMV-infected endothelial cells was increased significantly up to 39%. This increased adhesion was reduced with 64% by incubation with anti-IL-1. $F(ab)_2$ fragments of mAb ENA1 reduced the PMN adhesion to CMV-infected endothelial cells by 80%, $P<0{\cdot}05$. Thus incubations with anti-IL-1 or mAb ENA1 reduced the virus-induced PMN adhesion to endothelial cell monolayers to almost control values.

PMN adhesion through the CD18 receptor

The relation of the virus-induced adhesion with the CD18 antigen on the PMN was investigated. mAb IB4 (directed against the common beta chain of LeuCAM [CD18]) was added to the PMN suspension before testing the adhesion. The results show that pretreatment of PMN with saturating concentrations of mAb IB4 reduced the adhesion to non-infected and infected endothelial cell monolayers by 46% and 70%, respectively (Fig. 3). These values indicate that both control and virus-induced adhesion are mediated by CD18 and that virus infection results in an increased CD18 complex-dependent PMN adhesion.

Table 1. Expression of viral antigen (mAb H222) and activation antigen ELAM-1 (mAb ENA1) on CMV-infected endothelial cell monolayers measured with IF at different hours post-infection, as described in viral techniques. Data are presented as percentage of cells which gave a positive staining with mAb ENA1 or with mAb H222/microscopic area

Post-infection (hr)	% ENA1 antigen	% viral antigen
6	30	10
12	60	20
24	25	20

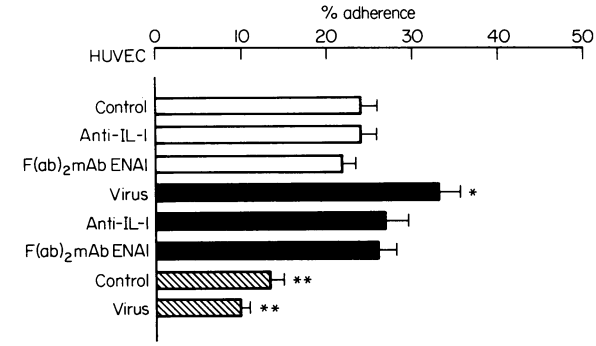

Figure 3. Effects of anti-IL-1 and $F(ab)_2$ fragments of mAb ENA1 on control and virus-induced adhesiveness. Endothelial cell monolayers were incubated, for 1 hr by 37°, in the presence of virus (closed bars) or supernatant derived from mock-infected monolayers (open bars), washed three times and then exposed to anti-IL-1 or $F(ab)_2$ mAb ENA1 for 23 hr at 37°. After washing twice, adhesion of untreated PMN or mAb IB4 (against CD18)-labelled PMN (hatched bars) was assessed using the ^{51}Cr method described in the Material and Methods. Controls were compared with virus-infected endothelial cells or with mAb IB4-labelled PMN. (*$P<0{\cdot}05$; **$P<0{\cdot}001$).

DISCUSSION

This study concerns the role of an activation antigen, reactive with mAb ENA1, in the interaction of PMN with CMV-infected endothelium. CMV infection of endothelium results in the appearance of an activation antigen ELAM-1 which is recognized by mAb ENA1. The expression of this antigen is maximal at 12 hr p.i. The expression of the activation antigen was optimal after 6 hr of incubation with IL-1. In both situations the ELAM-1 expression declined after maximal expression. Simultaneous to this activation antigen expression, the expression of viral antigens was measured. Since the two expression profiles show a similar time curve, a double IF was performed to test whether the observed CMV-induced activation antigen expression occurred in the virus-infected cells only. The results of these IF experiments indicate that in infected endothelial cell monolayers both ENA1- and CMV-positive cells do occur, but the cells reactive with mAb ENA1 do not express the CMV antigens. On a cellular level it was not possible to detect both signals (i.e. ENA1 and CMV antigens) on the same cells. This does not exclude the fact that CMV-infected cells can express the activation antigen on the surface themselves, but is probably due to the limitation of the method. These results can be explained by the fact that CMV infection of the cells does not induce ELAM-1 expression directly but indirectly through one or more other factors resulting in the activation antigen ELAM-1 expression on non-infected cells.

Since IL-1 incubation induces the ELAM-1 expression (Fig. 2), the possibility exists that IL-1 is involved in our system.

Although IL-1 production was not detected in the supernatant of infected monolayers, probably due to the fact that the amount of IL-1-producing cells was too low because only a maximum of 10% of the cells were infected,[10] the role of IL-1 is supported by two observations. First, the PMN adhesion occurred on infected but also on non-infected endothelial cells. Second, the incubation with anti-IL-1 resulted in an almost complete decline of the virus-induced ELAM-1 expression. The adhesion experiments demonstrate that this antigen recognized by mAb ENA1 is associated with the virus-induced PMN adhesion. This is emphasized by the observation that the incubation with anti-IL-1 also reduced the virus-induced PMN adherence. These results support the hypothesis that virus-induced PMN adhesion to endothelium is mediated by virus-induced IL-1 production, which in turn activates the endothelial cells and causes the expression of an antigen recognized by mAb ENA1. The inhibition of PMN adhesion, by $F(ab)_2$ fragments of mAb ENA1, indicates that the epitope reactive with mAb ENA1 is involved in the CMV-induced PMN adhesion.

These findings are supported by other reports. Several investigators have shown that stimulation of endothelial cells results in the production of IL-1-like activities.[32,33] This locally produced IL-1 acts on target cells, including endothelial cells, and is able, at relative low concentration, to stimulate the endothelial plasma membrane by which neutrophils, monocytes[18] and lymphocytes[19] avidly adhere. In this interaction, caused by IL-1, different types of molecules expressed on the surface of endothelial cells are involved, such as ICAM-1, ICAM-2 and ELAM-1.[34-36] Recent studies in our laboratory have shown that the expression of ICAM-1 is also increased in CMV-infected endothelium (published observations). In addition it is demonstrated that the PMN adhesion is not reduced completely after incubation with $F(ab)_2$ fragments of mAb ENA1. From these data it is evident that other antigens, as described above or the recently described GMP140 protein, are involved. The GMP140 expression, present on platelets and endothelial cells, changes markedly after cellular activation.[37] And as the domain organization of GMP140 is similar to that of ELAM-1, a possible role in neutrophil binding is suggested[38] which was recently confirmed by Geng *et al.*[21]

Furthermore, the virus-induced IL-1 production is not the only mediator of the CMV-induced enhancement of PMN adhesion, as shown by the incomplete reduction of adhesion using anti-IL-1. This suggests again that other triggers may be involved in the induction of PMN adhesion, like viral proteins,[39,8] and platelet-activating factor.[40,41]

Further, we studied if the PMN adhesion is mediated via the CD18 antigen present on the PMN membrane. The involvement of LFA-1, Mac-1, and p150,95 in the adhesion of leucocytes to endothelial cells has been established by inhibition experiments with mAb anti-CD18.[42] Our results suggest that adhesion of PMN to non-stimulated endothelial cells is dependent on the glycoprotein family (LeuCAM) expressed on the PMN membrane. Virus infection of endothelial cells up-regulates this CD18-dependent adhesion, which indicates that virus infection induces human endothelial cells to increase their adhesivity for PMN by a mechanism dependent on the common beta chain (CD18) of LeuCAM.

Finally, our observations document that virus infection modulates PMN adhesion to endothelial cells by changing the endothelial IL-1 secretion and the ELAM-1 expression (inhibitory effect on PMN adhesion of mAb ENA1). Furthermore these results provide new insights into how CMV-altered endothelium becomes attractive to granulocytes. However, since the other mechanism(s) is unknown, much remains to be explored to extend our understanding of the complex interaction of leucocytes with the endothelial cells during virus infection.

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