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Characterization of Influenza Virus-Induced Leukocyte Adherence to Human Umbilical Vein Endothelial Cell Monolayers[1]

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Abstract. The adherence of undifferentiated ^{51}Cr-labeled HL-60 (0.5×10^6 HL-60 cells/well) cells was monitored on influenza virus-infected HUVEC monolayers. Whereas only 3.0 ± 1.6% (n = 36) of HL-60 cells adhered to uninfected HUVEC, adherence was increased to 41.7 ± 2.2% (n = 6), 79.7 ± 1.2% (n = 6), 83.9 ± 0.7% (n = 6), and 84.4 ± 0.5% (n = 6) on HUVEC infected for 7 h at a MOI of 1, 3, 6, and 9, respectively. In comparison, HL-60 cell adherence increased to 35% when HUVEC monolayers were stimulated with LPS (0.2–20 μg) for 4 h. Increased adherence to infected HUVEC occurred at 5 h postinfection, peaked at 7 h, and was maintained at 24 h postinfection. Active virus and metabolically active endothelial cells were required to mediate the virus-induced adherence. E-selectin and ICAM-1 Ag were upregulated 78.3- and 4.1-fold, respectively, by LPS (0.02–20 μg, 4 h) whereas virus infection (7 h) only increased these proteins 2.6- and 1.4-fold with a MOI ≥ 16. Although the time courses of expression for both adhesion molecules after LPS treatment or virus infection were similar, the difference in the magnitude of upregulation suggests that virus-induced adherence is not a result of upregulation of E-selectin and ICAM-1. In contrast, surface expression of HA is involved in HL-60 cell adherence to virus-infected HUVEC because (1) the time course and magnitude of HA Ag expression paralleled the time course and magnitude of HL-60 cell adherence after virus infection of HUVEC; (2) HL-60 cell aggregates were absent on infected HUVEC monolayers in the presence of anti-HA; (3) HL-60 cells competed with RBC for infected endothelial cells stained for cellular HA Ag and (4) anti-HA abolished the virus-induced adherence. Furthermore, it appears that HL-60 cells are binding directly to HA because HL-60 cell adherence to a cell-free surface was increased if virus was prebound and neuraminidase treatment of HL-60 cells prevented the HL-60 cell adherence to influenza virus-infected endothelial monolayers. *Journal of Immunology*, 1993, 151: 310.

Leukocyte adherence to endothelial cells lining blood vessels is an integral part of an inflammatory response. Both in vivo (1) and in vitro (2–4) studies indicate that reactive oxygen species and proteolytic enzymes released at leukocyte-endothelial cell adherence sites produce endothelial cell injury. Endothelial cell injury, in turn, has been implicated in the development of various vascular disorders, including atherosclerosis, vasculitis, and adult respiratory distress syndrome (5–7). In some of these inflammatory diseases, the presence of viral particles, viral antigens, and viral DNA has suggested that viral infections play a role in the progression of vascular disease (5, 8–10). Further support for this possibility has come from in vitro observations that cultured endothelial cells infected with HSV, CMV, adenovirus, or polio virus develop an increased adhesiveness to phagocytic leukocytes (7, 11–18).

Whereas few studies have examined the role of endogenous endothelial cell adhesion molecules, such as ICAM-1, E-selectin, and P-selectin, in virus-induced leukocyte-endothelial cell adhesion, their roles in cytokine-induced leukocyte adhesion have been well characterized (19–24). Furthermore, little is known about the role of viral

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pathogen-derived molecules in either directly or indirectly mediating leukocyte-endothelial cell adhesion. In HSV-infected endothelial cells, the viral glycoprotein C molecule results in the local generation of thrombin, which then mediates the upregulation of P-selectin (25). Thus, in HSV-infected endothelial cells a viral glycoprotein indirectly mediates the increased leukocyte adherence.

In vitro influenza infection of endothelial cells also produces an increase in leukocyte adherence (26). Although the in vivo correlate of this observation is unclear, because influenza is commonly associated with respiratory epithelium, extrapulmonary manifestations have been reported. These manifestations include viremia (27–28) and, following fatal influenza pneumonia, recovery of the virus from the adrenal glands, heart, liver, meninges, and spleen (29–32). Thus, in vivo infection of endothelial cells is likely to occur during a disseminated influenza infection.

The purpose of this study was to further characterize influenza virus-induced leukocyte adherence to endothelial cell monolayers and to determine the mechanism underlying the increased adherence. We have demonstrated an increased adherence of HL-60 cells to influenza virus-infected HUVEC[3] monolayers as early as 5 h after infection. Whereas small increases in E-selectin and ICAM-1 Ag expression were noted in infected endothelial cells, these Ag played a minor role in the increased leukocyte adherence. Rather, our studies indicate that HL-60 cell adherence was mediated directly by the expression of the influenza virus glycoprotein HA on the surface of the infected endothelium.

Materials and Methods

Cell culture

Endothelial cells were dislodged from the vessel wall of the umbilical vein from human umbilical cord (Holy Cross Hospital, Bethesda, MD) by incubating with a 1% collagenase/PBS solution (Type II, Worthington Biochemical Corp., Freehold, NJ) for 15 min at 37°C. Cells were sloughed off by kneading the cord and flushing the lumen with MCDB107 medium (American Biorganics, Inc., N. Tonawanda, NY). Complete MCDB107 containing 10% heat-inactivated FCS (Hyclone Laboratories, Inc., Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (all from GIBCO Laboratories, Grand Island, NY), 100 μg/ml heparin (Sigma, St. Louis, MO), and 50 μg/ml endothelial cell growth supplement (H-Neurext, Upstate Biotechnology, Inc., Lake Placid, NY) was added to cells that were then centrifuged at 1500 rpm for 5 min to pellet cells. Pellets were resuspended in complete MCDB107, plated in 100-mm collagen-coated (Type II, Collaborative Research, Bedford, MA) tissue culture dishes, and placed in a 37°C, 95% air/5% CO_2-humidified incubator. Purity of our endothelial cell population was confirmed by the characteristic "cobblestone," nonoverlapping morphology of confluent monolayers (33, 34) and the presence of uniformly distributed acetylated low-density lipoprotein identified with the fluorescence probe 1,1′-diotadecyl-1-3-3-3′-3′-tetramethyl-indocarbocyanine perchlorate (Biomedical Technologies, Inc., Stoughton, MA) as previously described (35). Experimental data were obtained from HUVEC in their second to sixth passages, which were 1 to 2 days postconfluent.

HL-60 cells (American Type Culture Collection, Rockville, MD) were used in the undifferentiated state to assess leukocyte-endothelial cell adherence. The cell line was grown in suspension with RPMI 1640 (GIBCO) containing 10% nonheat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg/ml neomycin, and 2 mM glutamine. HL-60 cells used in the adhesion assay were in their 23rd to 27th passages.

mAb

Murine mAb H18/7 (IgG_{2a}, a gift from Dr. M. Gimbrone, Brigham & Women's Hospital, Boston, MA), binds to a functional epitope on the HUVEC surface protein ELAM-1 (E-selectin). Murine mAb 84H10 (IgG_{2a}), donated by Dr. S. Shaw (National Cancer Institute, Bethesda, MD), recognizes an epitope on the HUVEC surface protein ICAM-1. Murine mAb H17-L19 (IgG1), which blocks the RBC binding site on the globular head of the viral glycoprotein HA, was produced from a hybridoma provided by Dr. W. Gerhard (Wistar Institute, Philadelphia, PA). Control murine mAb, W6/32 (IgG_{2a}, Accurate Chemical & Scientific Corporation, Westbury, NY), which recognizes an HLA-A,B,C determinant constitutively expressed on HUVEC, was used as a nonrelevant binding antibody.

Virus preparation and infection

The WSN (H1N1) strain of influenza virus type A was grown in the MDCK cell line as previously reported (36). Stock virus was titered at 2–8 × 10^9 plaque-forming U/ml and stored in liquid nitrogen until needed. Endothelial cells were infected by adding influenza virus (MOI = 1, unless otherwise noted) in complete MCDB107 to HUVEC monolayers. After 1 h of adsorption, the medium was aspirated and rinsed once before fresh complete MCDB107 was added to each well.

Assessment of cell viability

Cell viability was assessed after virus infection and/or LPS treatment of the HUVEC monolayers by performing a cytotoxicity assay using a colorimetric kit (LK-100). Proteins

[3] Abbreviations used in this paper: HUVEC, human umbilical vein endothelial cell; HL-60, human promyelocytic leukemia cell; HA, hemagglutinin; MOI, multiplicity of infection; CPE, cytopathic effects; $TCID_{50}$, 50% tissue culture infectious dose.

Table I
Endothelial cell viability after virus infection and/or LPS treatment[a]

Treatment		% Cytotoxicity
Naive HUVEC (spontaneous release)		5.2 ± 0.7 (10)
LPS-treated HUVEC		3.9 ± 0.6 (5)
Virus-Infected HUVEC	7 to 9 h postinfection	24 h postinfection
MOI 0.1		28.2 ± 3.2 (5)*
MOI 1	3.9 ± 0.6 (9)	20.4 ± 1.2 (5)*
MOI 8	3.3 ± 0.9 (5)	30.3 ± 0.6 (5)*
MOI 1 + LPS 0.5 μg	2.7 ± 0.5 (5)	

[a] Data are expressed as the mean ± SEM with the number of observations in parenthesis. HUVEC monolayers were either mock infected, infected, or infected and LPS-treated. Cytotoxicity was assessed as described in the *Materials and Methods*.

The symbol * denotes a statistical difference between spontaneous release naive HUVEC and the test group ($p \leq 0.05$).

International, Rochester Hills, MI) that equates the release of lactate dehydrogenase to the number of injured cells. At various times after virus infection (MOI 0.1, 1, or 8) and/or 4 h after LPS treatment (0.5 μg) of HUVEC monolayers plated in 96-well plates, 100 μl of supernatant from each well was mixed with 100 μl of substrate mixture. After 30 min, color development was stopped with 1 N HCl, and absorbance was read at 492 nm wavelength on a Titertek ELISA plate reader (ICN Biomedicals, Inc., Costa Mesa, CA). Supernatants from control HUVEC were used to measure the spontaneous release of the enzyme, whereas supernatants from HUVEC exposed to a lysing reagent were used to determine the maximum release of the enzyme. The following equation was used to calculate percentage of cytotoxicity:

$$\% \text{ cytotoxicity} = \frac{\text{exp. abs} - \text{spont. abs}}{\text{max. abs.} - \text{spont. abs.}} \times 100$$

As shown in Table I, there was no measurable cytotoxicity 7 to 9 h after virus infection. In addition, a 4-h LPS treatment or a combined virus infection (7–9 h) and 4-h LPS treatment did not affect viability of the HUVEC monolayers. Cytotoxicity (20–30%) was observed only 24 h after virus infection of HUVEC. Therefore, experiments were performed 7 h after virus infection except when time courses were generated.

Virus infectivity titrations

To determine the susceptibility of HUVEC monolayers to influenza virus infection, infectivity was measured by quantitating the dilution of virus at which 50% of the infected cultures possessed CPE such as cell rounding, detachment, or death (37–38). HUVEC seeded in 96-well plates were infected with 100 μl of serial 10-fold dilutions of the virus in cold MCDB107. Wells with uninfected HUVEC were treated identically except that the medium contained no virus. After a 1-h adsorption period in a 37°C 5% CO_2-humidified incubator, the medium was aspirated and replenished, and cultures were returned to the incubator. Daily readings of CPE were recorded with the assay being terminated on day 7. The observed viral CPE was corroborated using a hemadsorption assay with human RBC (39) to detect surface viral HA protein. The $TCID_{50}$ was determined by the method of Reed and Muench (40). In viral titrations, the hemadsorption endpoint was compared with the CPE endpoint. Wells with visible CPE were always positive by hemadsorption, and wells with no signs of CPE were always negative.

Adhesion assay

HUVEC (30×10^4 cells/well) were seeded in collagen-coated 24-well plates 48 h before the confluent monolayers were virus infected. For comparison, other HUVEC monolayers were treated with LPS, a known promoter of leukocyte-endothelial cell interactions (41–44). At various times after infection or LPS treatment, ^{51}Cr-labeled HL-60 cells (0.5×10^6 cells/well) suspended in Dulbecco's MEM/5% FCS were added to HUVEC monolayers for 30 min at 37°C in a 5% CO_2-humidified incubator. Unbound HL-60 cells were aspirated, and the endothelial cell monolayers were washed five times with assay medium before the remaining adherent cells were lysed with 1 N NH_4OH. The lysate and a second wash with NH_4OH were transferred to vials for subsequent radioanalysis using an LKB 1282 COMPUGAMMA Counter CS (LKB Wallac, Turku, Finland). The percentage of HL-60 cell adherence was calculated as:

$$\% \text{ adherence} = \frac{\text{cpm test HL-60 cells} - \text{cpm } NH_4OH}{\text{cpm total HL-60 cells} - \text{cpm } NH_4OH} \times 100$$

The effects of endothelial-directed mAb on HL-60 adherence to uninfected or virus-infected HUVEC were determined by incubating HUVEC with saturating concentrations (90 μg/ml) of endothelial cell-directed mAb for 30 min at 37°C before and during the adhesion assay.

Detection of cell surface antigens

To measure surface Ag expression on HUVEC monolayers after influenza virus infection of LPS treatment, 2% paraformaldehyde-fixed (15 min at room temperature) HUVEC monolayers in collagen-coated 96-well plates were first incubated with PBS/1% BSA for 30 min at room temperature to block nonspecific binding. Each subsequent step of the ELISA was carried out at room temperature with three washes of PBS/1% BSA between steps. The fixed monolayers were incubated in turn with a saturating concentration of the test mAb and a peroxidase-conjugated

goat anti-mouse IgG (Sigma) for 1 h. *o*-Phenylenediamine (0.4 mg/ml)/0.012% hydrogen peroxide in a 0.05/0.025 M phosphate/citrate buffer, pH 5.0, was added to each well. Color development was stopped with 3 M H_2SO_4 at 20 min, and the OD was read at 492 nm wavelength. The degree of specific Ag expression was calculated by subtracting nonspecific binding of the secondary antibody from all test values.

Colocalization of cell aggregates and HA Ag

A modification of the hemadsorption assay was used to confirm that RBC and/or HL-60 cell aggregates were binding to infected endothelial cells. Infected HUVEC monolayers seeded on fibronectin-coated (Collaborative Research, Inc., Bedford, MA) glass coverslips (Bellco Biotechnology Vineland, NJ) were incubated for 30 min at 37°C in a 5% CO_2-humidified incubator with either RBC (human Type O, 0.5% in Gey's balanced salt solution), HL-60 cells (0.5×10^6 cells), or medium lacking cells. Cultures were rinsed of nonadherent cells, and then the second cell type was added to some cultures for 30 min before cultures were washed of nonadherent cells. The remaining endothelial cell monolayers with adherent RBC and/or HL-60 cells were fixed with 2% paraformaldehyde in PBS (7.4) for 30 min on ice. Cellular HA Ag was fluorescently monitored by permeabilizing the fixed HUVEC monolayers in −20°C acetone for 3 min before rinsing in PBS/50 mM NH_4Cl and storing overnight at 4°C. The monolayers were then washed in PBS/1% BSA for 30 min at room temperature, incubated with intact anti-HA (H17-L19, 1:20) for 1 h at room temperature, rinsed with PBS/1% BSA, and incubated with a rhodamine-conjugated goat anti-mouse IgG (1:100, Sigma). After 1-h incubation at room temperature in the dark with the secondary antibody, HUVEC monolayers were washed, monitored for fluorescence with rhodamine optics (546 to 610 nm excitation/590 nm emission/580 nm dichroic mirror, Carl Zeiss, Inc., Thornwood, NJ), and photographed on Ektachrome Tungsten 160 film (Eastman Kodak Co., Rochester, NY).

Data analysis

To test the effect of virus infection of HUVEC monolayers on HL-60 cell adherence and Ag expression the Student's *t*-test was used. The symbol * denotes a statistical difference ($p \leq 0.05$) between test and corresponding control groups.

Results

Susceptibility of HUVEC monolayers to influenza virus infection

The ability of influenza virus to infect HUVEC monolayers and produce CPE was monitored over a 7-day period.

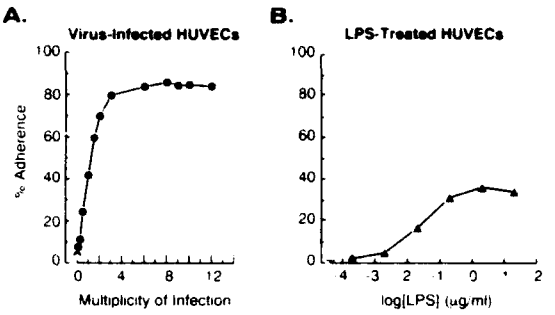

FIGURE 1. Dose response of HL-60 cell adherence to HUVEC monolayers (A) influenza virus infected for 7 h or (B) LPS treated for 4 h. HUVEC monolayers were exposed to various concentrations of virus or LPS, and a standard adhesion assay was performed as described in *Materials and Methods*. Each point represents the mean ± SEM of six replicates in a representative experiment of three separate experiments. In this graph and all subsequent graphs, error bars are omitted when smaller than the size of the symbol. The *open symbols* in all figures indicate control HUVEC.

Endothelial cytoplasmic vacuolization was detected in all HUVEC cultures ($n = 8$) infected with a 10^{-6} dilution on day 3 after infection, whereas cellular detachment was evident on days 4 to 7 with that same dilution. However, only 3 of 8 wells and 1 of 8 wells showed evidence of CPE on days 3 to 7 for HUVEC cultures infected with 10^{-7} and 10^{-8} dilutions, respectively. A $10^{-5.6}$ $TCID_{50}$/ml dilution was calculated to be the dilution at which 50% of the HUVEC cultures would possess CPE. In comparison, MDCK epithelial cells, which are known to fully support influenza virus replication, have a 10^{-8} $TCID_{50}$/ml with a comparable viral pool.

Characterization of HL-60 cell adherence to influenza virus-infected or LPS-treated HUVEC

The adherence of HL-60 cells to uninfected control HUVEC monolayers was 3.0 ± 1.6% (Fig. 1 A). In contrast, HL-60 cell adherence to influenza virus-infected HUVEC monolayers monitored 7 h postinfection was increased in a concentration-dependent manner. Adherence was saturated with 83% of HL-60 cells binding to endothelial monolayers infected with a MOI ≥4 (Fig. 1 A). Microscopic inspection of monolayers indicated that HL-60 cells bound in both singlets and aggregates to infected HUVEC monolayers. A similar virus-induced adherence occurred with cAMP-differentiated HL-60 cells and freshly isolated human neutrophils (data not shown). This virus-induced adherence differed from the adhesion produced by treating HUVEC for 4 h with saturating concentrations of LPS, where HL-60 cells adhered as singlets, and adherence was increased to only 35% (Fig. 1 B).

HL-60 cell adherence to LPS-treated HUVEC reached maximal levels by 3 h, remained elevated through 7 h, and

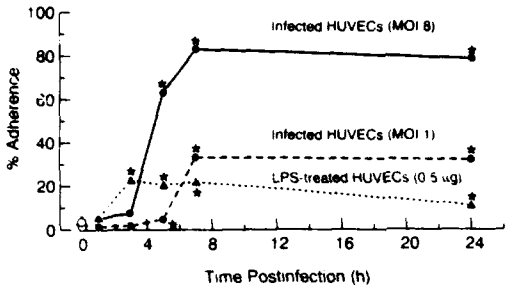

FIGURE 2. Time course of HL-60 cell adherence to virus-infected or LPS-treated HUVEC monolayers. HL-60 cell adherence to HUVEC monolayers was monitored at various times after virus infection (MOI 1 or 8) or LPS treatment (0.5 μg). Each point is the mean ± SEM of six replicates in a representative experiment of three separate experiments.

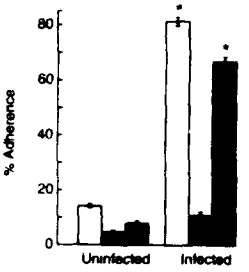

FIGURE 3. Effect of fixation on HL-60 cell adherence to virus infected HUVEC monolayers. The adhesion assay was performed with HUVEC monolayers that were either mock- or virus-infected (MOI 8) before (*solid bars*) or after (*striped bars*) fixing with 2% paraformaldehyde for 5 min and washed twice. The *open bars* represent unfixed HUVEC monolayers. A small decrease in antigenicity produced by fixation probably caused the slight decline in HL-60 cell adherence to fixed uninfected HUVEC monolayers. Each *bar* is the mean ± SEM of six replicate wells in a typical experiment of three separate experiments.

decreased 49% after 24 h of LPS treatment (Fig. 2). Increased adherence to infected HUVEC occurred at 5 h postinfection, peaked at 7 h, and remained at maximal levels 24 h after infection regardless of the virus titer used (Fig. 2).

To determine if viable influenza virus was required to stimulate HL-60 cell adherence to HUVEC monolayers, the adhesion assay was performed with active and UV-inactivated virus. Exposure of the virus to UV light for 30 min before addition to HUVEC monolayers abolished the increase in HL-60 cell adherence (data not shown). Furthermore, HL-60 cell adherence to paraformaldehyde-fixed HUVEC that were subsequently infected with active virus failed to show an increased adherence (Fig. 3, striped bar). In contrast, HUVEC fixed 7 h after infection with active virus displayed an 8.6-fold increase in leukocyte adherence (Fig. 3, filled bar). Fixing HL-60 cells before performing the adhesion assay with infected HUVEC did not prevent the virus-induced adherence (data not shown). Thus, the interaction of metabolically active endothelial cells and live virus was required for the increased HL-60 cell adherence, while metabolically active HL-60 cells were not required.

Surface expression of endothelial adhesion molecules

The dose-response of E-selectin and ICAM-1 surface Ag expression induced by either influenza virus infection or LPS treatment is shown in Figure 4. While the ICAM-1 Ag was expressed constitutively on the surface of uninfected endothelial cells, the E-selectin Ag was not. ICAM-1 and E-selectin expression was increased 1.1- and 1.4-fold, respectively, with an MOI of 1, and maximally increased 1.3- and 2.6-fold with an MOI ≥ 16 (Fig. 4 A). As a comparison, the surface expression of ICAM-1 and E-selectin was monitored after a 4-h treatment of HUVEC with LPS (Fig. 4 B). ICAM-1 and E-selectin Ag increased by 4.1- and 78.3-fold, respectively, after exposure to LPS (0.02–20 μg). Constitutively expressed HLA-A,B,C Ag, monitored by the

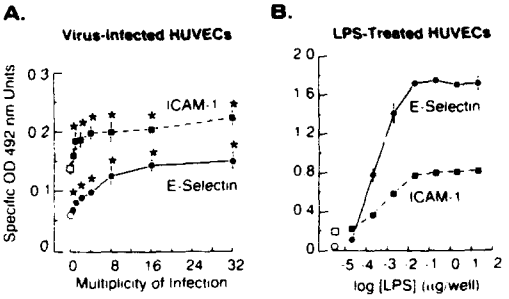

FIGURE 4. Dose response of surface Ag expression on HUVEC monolayers (A) virus infected for 7 h or (B) LPS treated for 4 h. HUVEC were exposed to various concentrations of virus or LPS, and surface ICAM-1 and E-selectin expression was quantitated by the ELISA assay described in *Materials and Methods*. Note the difference in the ordinate scales. Each *point* is the mean ± SEM of quadruplicate wells of a representative experiment of two separate experiments. Ag expression for both adhesion molecules was significantly greater than control Ag expression (*open symbols*) at LPS concentrations ≥ 0.0002 μg.

W6/32 antibody of the same isotype, was not altered by LPS treatment or virus infection of HUVEC (data not shown).

The time course of Ag expression of both adhesion molecules after virus infection or LPS treatment was followed using a virus titer and an LPS dose shown to induce E-selectin and ICAM-1 (Fig. 5). E-selectin surface Ag was induced on virus-infected HUVEC as early as 1 h postinfection, peaked between 5 and 7 h postinfection, and began to decrease at 24 h postinfection (Fig. 5 A). Virus-induced ICAM-1 expression did not begin until 5 h and continued to increase by 24 h postinfection. A comparison of the data

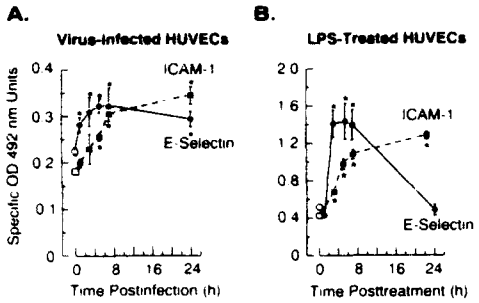

FIGURE 5. Time course of surface antigen expression on HUVEC monolayers (A) virus infected with an MOI of 1 or (B) treated with 0.5 μg LPS. ICAM-1 and E-selectin expression was quantitated at various times after virus infection or LPS treatment of HUVEC monolayers. Note the difference in the ordinate scales. Each point is the mean ± SEM of quadruplicate wells in a typical experiment of three separate experiments.

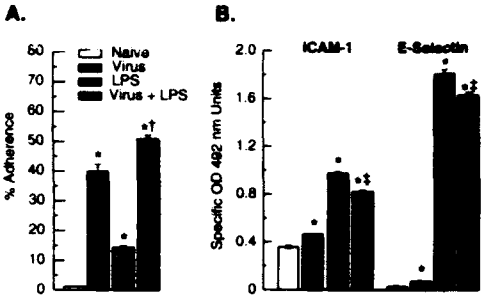

FIGURE 6. (A) HL-60 cell adherence to and (B) surface Ag expression on HUVEC monolayers that were either control (naive), virus infected (MOI 1), LPS treated (0.5 μg), or infected and LPS treated. The combined treatment protocol consisted of HUVEC monolayers being infected for 5 h before they were LPS treated for 4 h. Each bar is the mean ± SEM of (A) six replicates and (B) four replicates of two separate experiments. * denotes a statistical difference ($p \leq 0.05$) between test and corresponding control groups. † signifies a significant difference between the combined treatment group and the infected group ($p \leq 0.05$). ‡ indicates a statistical difference between the combined treatment group and the LPS group ($p \leq 0.05$).

on virus-infected HUVEC (Fig. 5 A) to that obtained from LPS-treated HUVEC (Fig. 5 B) indicates that the magnitude of the increase in endothelial adhesion molecules induced by virus infection follows a similar time course but was less than 30% of the expression after LPS treatment.

It was possible that influenza virus infection of HUVEC reduced host protein synthesis, thereby producing only small increases in E-selectin and ICAM-1 Ag. To test this possibility, HL-60 cell adherence and adhesion molecule Ag expression was monitored after a combined virus infection and LPS treatment protocol. Five h after HUVEC monolayers were infected with influenza virus, the infected cells were exposed to LPS (0.5 μg) for 4 h. This time point was chosen because at 5 h postinfection cells will be actively synthesizing and packaging viral proteins (39, 45). As shown previously, HL-60 cell adherence to HUVEC monolayers was enhanced 36-fold after virus infection, and 4 h of LPS treatment alone produced a 13-fold increase in adherence (Fig. 6 A). HUVEC exposed to a combination of influenza virus and LPS, however, showed an additive effect with a 46-fold increase in HL-60 cell adherence compared to control conditions (Fig. 6 A), indicating that prior virus infection did not significantly inhibit LPS-induced HL-60 cell adherence.

ICAM-1 and E-selectin Ag expression was quantitated under the same experimental conditions. While virus infection alone only increased ICAM-1 and E-selectin expression by 1.3- and 2.6-fold, respectively, LPS treatment produced a 2.7- and 67-fold respective increase in these surface proteins (Fig. 6 B). ICAM-1 and E-selectin Ag expression continued to be upregulated by LPS-treatment after virus infection of HUVEC. However, the actual 2.3- and 60.2-fold respective increase in ICAM-1 and E-selectin Ag following the combined treatment protocol was 43 and 14%, respectively, lower than the expected values would have been if the effects of LPS treatment and viral infection were additive. Nonetheless, the small reduction in ICAM-1 and E-selectin levels by infection cannot account for the failure of virus infection to maximally upregulate these molecules.

Role of the influenza viral glycoprotein HA in virus-induced adherence

The hemadsorption assay and immunofluorescence techniques demonstrated that HL-60 cells bind and aggregate to endothelial cells that stain positive for the HA Ag (Fig. 7). In fact, HL-60 cells competed with RBCs for binding to HA-positive endothelial cells, suggesting that HL-60 cells bound specifically to the HA protein on the surface of the infected HUVEC (Fig. 7 C). To determine if sialic acid residues on the surface of HL-60 cells interacted with HA protein budding on the infected HUVEC monolayer, we treated HL-60 cells with neuraminidase to cleave sialic acid residues and quantitated adherence to virus-infected HUVEC monolayers. While HL-60 cell adherence to uninfected HUVEC monolayers was not affected by prior neuraminidase treatment, virus-induced adherence was inhibited by 98% (Fig. 8).

As illustrated in Fig. 9, HA Ag expression was monitored on HUVEC monolayers 7 h after infection with various titers of influenza virus. As expected, there was minimal HA expression on the surface of uninfected HUVEC monolayers. However, infection of the endothelial cell monolayers dose-dependently increased HA Ag with a maximal 12.7-fold increase at an MOI ≥ 16, which was shown previously to saturate HL-60 cell adherence. HA expression

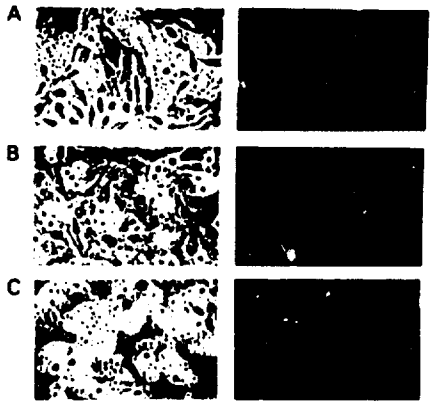

FIGURE 7. Bright field (*left panel*) and fluorescent (*right panel*) micrographs examining colocalization of (*A*) RBC, (*B*) HL-60, or (*C*) RBC + HL-60 aggregates and cellular HA Ag 7 h after influenza virus infection (MOI 24) of HUVEC monolayers. Virus-infected HUVEC monolayers were incubated with RBC, HL-60 cells, or RBC first and then HL-60s or vice versa before they were fixed and stained for cellular HA Ag as described in *Materials and Methods*. *Bar* = 45 μm.

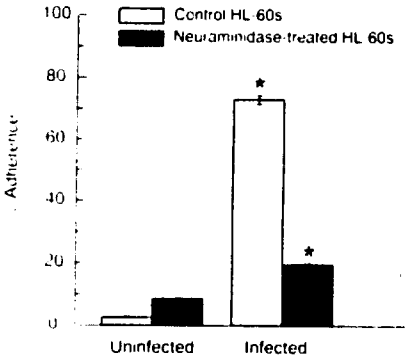

FIGURE 8. Effect of neuraminidase treatment of HL-60 cells on adherence to virus-infected HUVEC monolayers. HL-60 cells were exposed to neuraminidase (0.1 U, from *Vibrio cholerae*, Sigma, St. Louis) in RPMI/5% FCS for 30 min at 37°C with gentle agitation, washed several times, and then used in the adhesion assay to quantitate adherence to HUVEC monolayers infected (MOI 1) for 7 h. Each bar is the mean ± SEM of five to six replicate wells in a representative experiment of two separate experiments.

was similar with titers as high as an MOI of 90 (data not shown). This viral protein was not apparent on infected HUVEC until 5 h postinfection, peaked at 7 h, and remained maximal at 24 h postinfection, which paralleled the time course of HL-60 cell adherence to the infected endothelium (Fig. 10). HA Ag expression was not evident on HUVEC treated with LPS for 4 h at concentrations that maximally induced expression of E-selectin and ICAM-1 Ag (data not shown).

To directly demonstrate the role of surface HA in the virus-induced adherence, two experimental protocols were performed. First, uninfected and infected HUVEC were incubated with $F(ab')_2$ fragments of anti-HA, intact anti-E-selectin, anti-ICAM-1, and/or anti-HLA before and during the adhesion assay. The presence of anti-HA alone blocked the virus-induced HL-60 cell adherence by 95% while it had no effect on basal adherence (Fig. 11). In addition, no HL-60 cell aggregates were visible on infected HUVEC monolayers in the presence of anti-HA. While the combined presence of antibodies against HA, E-selectin, and ICAM-1 inhibited the virus-induced adherence by 96.5%, there was no specific effect of anti-E-selectin and anti-ICAM-1 on induced adherence since exposure to these two antibodies inhibited virus-induced adherence to the same extent (19%) as the nonrelevant binding anti-HLA (Fig. 11).

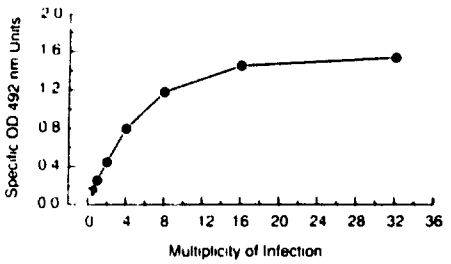

FIGURE 9. Dose response of surface HA antigen expression on infected HUVEC monolayers. Seven hours after exposure of HUVEC monolayers to various titers of influenza virus, monolayers were fixed, and HA Ag expression was quantitated using H17-L19 (anti-HA) in the ELISA assay described in *Materials and Methods*. Each point is the mean ± SEM of quadruplicate wells in a representative experiment of two separate experiments. HA Ag expression was significantly different from control Ag expression at MOI ≥ 1.

Second, the adhesion assay was performed with ^{51}Cr-labeled HL-60 cells in the absence of HUVEC monolayers on polylysine-coated wells without and with bound influenza virus. Adherence of HL-60 cells to polylysine-coated wells was minimal with bound singlets (Fig. 12). However, when HL-60 cells were added to wells that had the virus bound to the surface, there was a threefold increase in adherent HL-60 cells, with some cells binding as aggregates (Fig. 12).

Discussion

Viral infections, including influenza virus infections, have been associated with leukopenia (46–50). One of the possible causes for this virus-induced leukopenia may be adherence of circulating leukocytes to virus-infected endothelial cells lining blood vessels since as both enteroviruses that cause an acute lytic infection and adenoviruses that produce a chronic, slowly lytic infection of endothelial cell monolayers also have been shown to enhance granulocyte adherence to endothelial cell monolayers (11–12). In this study we demonstrate that infection of cultured endothelial

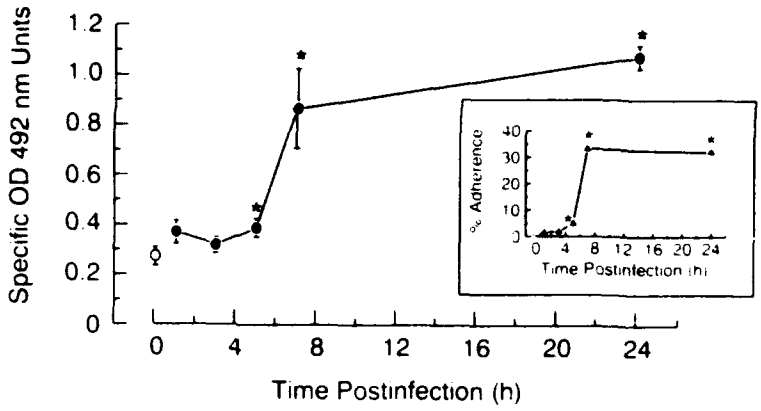

FIGURE 10. Time course of HA Ag expression on infected HUVEC monolayers. At various times after virus infection (MOI 1) of HUVEC monolayers, surface HA Ag expression was measured. Each point is the mean ± SEM of quadruplicate wells in a representative experiment of two separate experiments. Inset: Time course of HL-60 cell adherence to virus-infected (MOI 1) HUVEC monolayers (same as in Fig. 2).

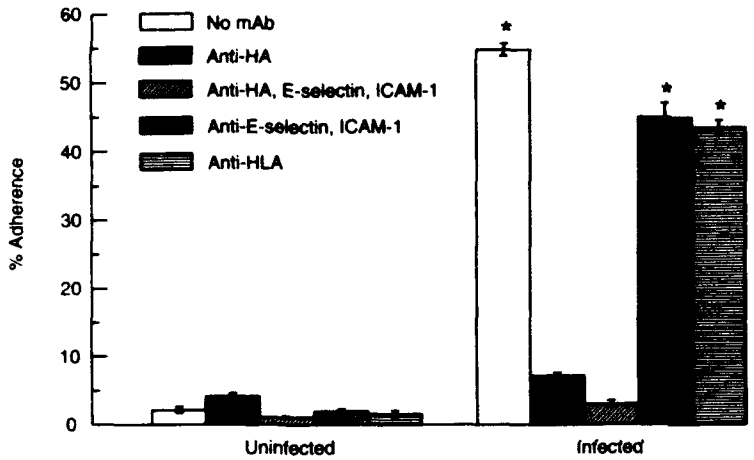

FIGURE 11. Effect of endothelial-directed antibodies on HL-60 cell adherence to virus-infected HUVEC monolayers. Uninfected or infected (MOI 1, 7 h postinfection) HUVEC monolayers were exposed to no antibody, anti-HA (H17-L19), anti-E-selectin (H18/7), anti-ICAM-1 (84H10), and/or anti-HLA (W6/32) before and during the adhesion assay. Each *bar* is the mean ± SEM of four to five replicate wells in a representative experiment of two separate experiments.

cells with a common human pathogen, influenza virus type A, also promotes leukocyte adherence and that the molecule underlying the increased adherence is the influenza viral protein HA expressed on the surface of infected endothelial cells.

In contrast to the Victoria/75 (H3N2) strain of influenza virus type A, which did not infect human venous or bovine arterial endothelium at a low virus titer (MOI 0.1–0.3) (51), the WSN (H1N1) strain in our study produced a slowly lytic infection in HUVEC as evidenced by the time of onset of CPE. Hemadsorption of RBC and immunofluorescence indicated that most of the endothelial cells were infected with virus under our experimental conditions. Moreover, we have shown previously, using electron microscopic techniques, that this strain of influenza buds from the apical surface of endothelial cells (52). The increase in HL-60 cell adherence to infected endothelium required metabolically active endothelial cells because fixing the HUVEC monolayers before exposing them to the virus-containing medium abolished the increase in HL-60 cell adherence. Conversely, fixing HL-60 cells did not alter the virus-induced adherence. A similar inhibition of induced leukocyte adherence occurs when endothelial cells are fixed before cytokine or LPS treatment (53–54).

The time course for HL-60 cell adherence to influenza virus-infected HUVEC was found to be similar to time courses of leukocyte adherence to endothelial cells infected with other viruses. HL-60 cell adherence to endothelial cell monolayers was modulated by influenza virus infection with an increase beginning at 5 h, peaking at 7 h, and lasting 24 h postinfection. Infection of endothelial cells with herpes viruses has been shown to enhance human neutrophil adherence as early as 4 h postinfection, with plateaus between 18 and 32 h postinfection (7, 13, 15, 17). Increased monocyte adherence to endothelial cells also occurs within 4 h of exposure to HSV 1 (16–17, 25) and was observed 25 h after either HSV (25) or CMV infection (17).

Influenza virus infection produced a robust 28-fold increase in HL-60 cell adherence under similar infection protocols (MOI 24) or with lower virus titers (MOI 1, ninefold increase) compared to the small twofold to threefold increase in leukocyte adherence observed in endothelial cells infected with CMV (17) and HSV (13, 25). This discrepancy in magnitude of response between our findings and

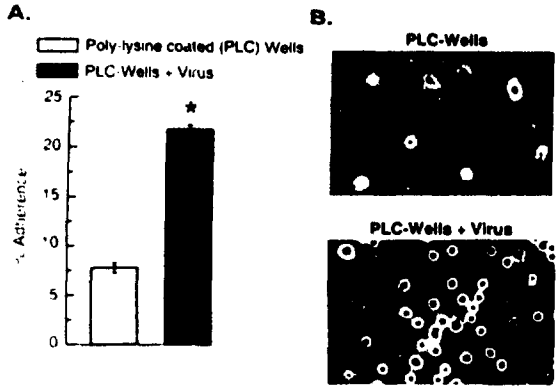

FIGURE 12. HL-60 cell adherence to polylysine-coated wells in the absence and presence of bound influenza virus. Wells were coated with polylysine (1 mg/ml) in Hank's balanced salt solution for 5 min, rinsed with deionized water, and allowed to dry. In some wells a high titer of virus (MOI 100) was added for 30 min at 37°C. After removing unbound virus, ^{51}Cr-labeled HL-60 cells (2×10^5 cells) were added to the wells for the adhesion assay to proceed as described in *Materials and Methods*. (*A*) Each *bar* is the mean ± SEM of five replicate wells in a representative experiment of two separate experiments. (*B*) Bright field micrographs of HL-60 cells bound to (*upper panel*) polylysine-coated wells and (*lower panel*) virus- and polylysine-coated wells. Same scale as in Fig. 7.

other studies may reflect the different leukocytes used in the various studies inasmuch as human neutrophils, monocytes, U937 cells, and HL-60 cells may exhibit different surface integrin profiles (55–57). Alternatively, these differences may be due to different mechanisms by which viral pathogens enhance leukocyte adherence. For example, the 1.4-fold increase in neutrophil adherence to HUVEC infected with CMV appears to be due, in large part, to the upregulation of surface ELAM-1 (E-selectin), because it was largely inhibited by an antibody against ELAM-1 (18). In contrast, both a soluble factor released from HSV-infected HUVEC (17) and a thrombin-dependent increase in GMP-140 (P-selectin) on the surface of infected HUVEC (25) have been implicated in the HSV-induced enhancement of monocyte binding.

In our studies, influenza virus infection of HUVEC monolayers produced small increases in two endothelial adhesion molecules, E-selectin and ICAM-1. While the magnitude of these increases was smaller, the time courses of upregulation of these adhesion molecules were similar to that produced after LPS or cytokine stimulation of HUVEC monolayers (41–44). However, the inability of antibodies against E-selectin and ICAM-1 to specifically block the increase in HL-60 cell adherence strongly suggests that these adhesion molecules play only a minor role or no role in the influenza virus-induced adherence and that other endothelial cells or viral proteins are involved. It is noteworthy that although ICAM-1 expression is also increased on parainfluenza virus-infected airway epithelial cells, an antibody against ICAM-1 has no significant effect on parainfluenza-induced neutrophil adherence (58). In HSV-infected HUVEC, neutrophil adherence is indirectly dependent on the surface expression of the herpes virus glycoprotein. That is, the surface expression of the herpes virus glycoprotein C induces the local generation of thrombin and subsequent upregulation of GMP-140 (13, 25). It is unlikely that GMP-140 upregulation plays a role in HL-60 cell adherence to influenza-infected HUVEC because previous studies have demonstrated that, unlike neutrophils, undifferentiated HL-60 cells do not bind to GMP-140 (59).

Rather than implicating the upregulation of an endogenous endothelial cell adhesion molecule, several observations indicate that the expression of the influenza virus glycoprotein HA on the endothelial surface mediates the increased binding of HL-60 cells to infected HUVEC. First, the time course and dose response for HA Ag expression parallels that of HL-60 cell adherence to infected HUVEC monolayers. Second, HL-60 cell aggregates were absent on infected HUVEC monolayers in the presence of anti-HA. Finally, anti-HA abolished HL-60 cell adherence to influenza virus-infected endothelial monolayers. Furthermore, HA appears to serve directly as a binding site for HL-60 cells, inasmuch as leukocyte adherence to a cell-free surface was increased if virus was prebound, and the ability of neuraminidase treatment of HL-60 cells to cleave sialic residues actually prevented the virus-induced adherence. Thus, like HSV-infected HUVEC, a viral protein is also involved in the influenza virus-stimulated increases in HL-60 cell adherence, but, unlike HSV-infected endothelial cells, surface expression of a viral protein directly mediates leukocyte adherence.

We have described a similar direct binding between leukocytes and the viral protein HA on epithelial cells (MDCK) infected with either the same WSN (H1N1) strain (60) or the A/PR8 (H1N1) strain of influenza virus (unpublished data). While the interactions of influenza with epithelial cells, and in particular the respiratory epithelium, have been well studied, relatively little is known about the effect of influenza virus on other tissues in the body. In vitro studies indicate that neutrophils, which accumulate during the early stages of an influenza infection, are capable of transporting influenza virions on the surface of and within phagocytic vacuoles from the luminal to the abluminal surface of an epithelium, and thus, may play a role in the spread of infection (61). Viremia has been documented in individuals in the 1 to 3 day incubation period before the onset of symptoms (27–28) and in individuals with uncomplicated influenza infection (62) as well as severe influenza pneumonia (30–32). During viremia and disseminated infection, it is likely that influenza infects the endothelia lining the vessels of the respiratory system and of other organs.

In vivo, influenza virus binding to sialic acid residues may mediate leukocyte rolling and/or stable adhesion. Leukocyte rolling, an early step in the inflammatory response, can be mediated by GMP-140 binding to a sialylated carbohydrate ligand with a Lewis x component (63) and presumably occurs because lectin-carbohydrate interactions possess fast on-and-off rates in the range of 10^5 to 10^6 M-1 s-1 and 2.3 to 56 s-1, respectively (64–66). Because very fast kinetics have been described for the interaction of influenza virus with HL-60 cells and other cultured cells ($k_{+1} > 10^{10}$ M-1 s-1 and $k_{-1} \leq 0.004$ s-1; (67)), and the K_D of virus binding to sialic acid residues is ≥ 1 mM (68 to 69) influenza virus infection of endothelial cells in vivo may similarly mediate leukocyte rolling. In our study, weak binding (sensitive to vigorous wash steps) was observed between HL-60 cells and influenza virus in a cell-free system. However, we observed a tighter binding (maintained after several washes) between HL-60 cells and virus-infected endothelial cells suggesting that an additional mechanism mediates this interaction. In contrast to RBC adherence, raising the temperature from 4°C to 37°C increases HL-60 cell adherence (data not shown) and neutrophil binding (39) to endothelial and epithelial cell monolayers, respectively. This suggests that the interaction between HA and its sialylated ligand on leukocytes is different from the binding between HA and RBC. Therefore, it is plausible that under flow conditions virus infection mediates not only HA-related leukocyte rolling, but subsequent facilitation of a more stable integrin-like adhesion.

In summary, we have demonstrated a time- and concentration-dependent increase in HL-60 cell adherence to influenza virus-infected endothelial cell monolayers that is predominantly due to HA, the newly expressed surface viral protein. These findings indicate that viral proteins can directly enhance leukocyte binding to infected cells. The findings also suggest that the expression of viral proteins on virus-infected cells may be an important step in virally mediated endothelial injury and the subsequent development of inflammatory responses.

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