

Early E-Selectin, VCAM-1, ICAM-1, and Late Major Histocompatibility Complex Antigen Induction on Human Endothelial Cells by Flavivirus and Comodulation of Adhesion Molecule Expression by Immune Cytokines

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Expression of E-selectin (ELAM-1, CD62E) on human umbilical vein endothelial cells significantly increased 30 min postinfection with the flavivirus West Nile virus (WNV), was maximal by 2 h postinfection, and declined to baseline levels within 24 h. Expression of ICAM-1 (CD54) and VCAM-1 (CD106) was significantly increased by 2 h and maximal at 4 h after infection. P-selectin (CD62P) expression was unaffected by WNV. Upregulation occurred earlier than that caused by tumor necrosis factor alpha (TNF- α) or interleukin 1 (IL-1) and could not be inhibited by neutralizing TNF- α , IL-1 α , or alpha/beta interferon (IFN- α/β) antibodies, suggesting a direct, virus-mediated phenomenon. TNF- α significantly enhanced WNV-induced increases in E-selectin, P-selectin, ICAM-1, and VCAM-1 expression, while IFN- γ enhanced WNV-induced ICAM-1 expression. In contrast, IL-4 abrogated WNV-induced E-selectin expression increases but acted in synergy with WNV to increase P-selectin and VCAM-1 expression. WNV increased the expression of class I and II major histocompatibility complex antigens (MHC-I and MHC-II, respectively) at 24 and 72 h, respectively. IFN- γ , TNF- α , or IL-1 acted in synergy with WNV to produce greater increases in MHC-I expression than WNV or cytokines alone, while IFN- α/β or IL-4 had no effect. MHC-II induction in cytokine-treated, WNV-infected cells was similar to that caused by cytokines alone. Neutralizing IFN- α/β antibody inhibited WNV-induced MHC-I expression by 30% at 24 h and by 100% by 72 h. The differential kinetics of modulation suggest sequential adhesion of leukocyte subpopulations to infected endothelial cells, which may be important in initial viral spread in vivo.

Flaviviruses are arthropod-borne, plus-strand RNA viruses, many of which are neurotropic although capable of infecting a wide variety of cells. Despite the pathological and socioeconomic importance of these viruses, knowledge of the immunological mechanisms occurring during flavivirus infection is limited. We have shown that flavivirus infection increases functional expression of class I and II major histocompatibility complex (MHC-I and MHC-II, respectively) antigens and intercellular adhesion molecule-1 (ICAM-1, CD54) in murine and human cells in vitro (1, 2, 11, 23, 24, 32, 52, 53) and in vivo (21) via a direct, virus-mediated mechanism. Since increased expression of these molecules increases the efficiency of recognition of and adhesion between T cells and infected target cells (11, 14, 26, 43, 57, 64), flavivirus-induced increases in the expression of these ligands seem paradoxical in terms of virus survival.

In flavivirus infection, the development of neuropathology is related to the level of viremia (41, 66), suggesting a role for endothelium in the transmission of infection across the blood-brain barrier. Much recent interest has been focused on leukocyte-endothelial cell adhesion mediated by inflammatory cytokines via increased adhesion molecule expression. E-selectin (CD62E), a ligand for carbohydrate sLe^x (3, 7) found on monocytes (30) and lymphocytes, including memory T cells (54) and NK cells (45), functions as a "rolling receptor" for leukocyte adhesion and is transcriptionally induced by the inflammatory cytokines interleukin 1 (IL-1) and tumor necrosis

factor alpha (TNF- α) (4, 28, 29, 31, 67). In contrast, P-selectin (CD62P) is stored in the Weibel-Palade bodies in endothelial cells and released quickly on exposure of these cells to thrombin (58), histamine, and complement proteins (15, 16). It may also be transcriptionally induced by TNF- α or IL-4 (67, 69). P-selectin is a rolling receptor for neutrophils (37) as well as for CD4⁺ lymphocytes (34). Vascular cell adhesion molecule-1 (VCAM-1, CD106), involved in the firm adhesion of eosinophils and other leukocytes via the VLA-4 receptor, can be induced by IL-1, TNF- α , IL-4, and IL-13 (6, 43, 50, 59, 63). ICAM-1 (CD54) is strongly upregulated by IL-1, TNF- α , and gamma interferon (IFN- γ) and contributes to cell-cell interactions through its counterpart, LFA-1 (12, 13, 36, 46, 48), on leukocytes. Some cytokines inhibit adhesion molecule induction by others. IL-4 inhibits the increased expression of E-selectin induced by IL-1 or TNF- α and the increased ICAM-1 expression induced by IL-1, TNF- α , or IFN- γ (47, 62). However, combinations of apparently opposing cytokines, such as TNF- α and IL-4, may act in synergy, e.g., in the induction of VCAM-1 expression on human umbilical vein endothelial cells (HUVEC) (44).

Cytomegalovirus (CMV) upregulates E-selectin and increases neutrophil-endothelial cell adhesion, but this occurs via IL-1 release (51, 56, 68). Cytokine release from infected cells is also the likely mechanism mediating increases in ICAM-1, VCAM-1, and MHC-I expression by measles virus (27), influenza virus (9), reovirus (8), simian immunodeficiency virus (49), and CMV (51).

In this study, we investigated the effects of flavivirus infection, with and without concomitant immunomodulatory cytokine exposure, on adhesion molecule and MHC expression on

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endothelial cells, since this influences the attachment and migration of leukocytes to the site of infection *in vivo*. We show that flavivirus directly induces increased E-selectin expression on HUVEC 30 min after infection and increased VCAM-1 and ICAM-1 expression within 2 h. Modulation was enhanced or inhibited by the simultaneous addition of inflammatory and Th1 or Th2 cytokines. In contrast to flavivirus induction on other cells, increased MHC-I and MHC-II expression occurred only at 24 and 72 h after infection, respectively, and, in the case of MHC-I, evidently occurred via both direct and indirect mechanisms.

MATERIALS AND METHODS

Antibodies. The following monoclonal antibodies were used as primary antibodies. Clone H18/7, mouse immunoglobulin G2a (IgG2a) (anti-ELAM-1, anti-E-selectin, anti-CD62E), and clone E1/6, mouse IgG1 (anti-VCAM-1, anti-CD106), were purchased from Becton Dickinson (Sydney, New South Wales, Australia). Clone 84H10, mouse IgG1 (anti-ICAM-1, anti-CD54), was purchased from Immunotech Soborb, Adelaide, S.A., Australia). Clone AK4, mouse IgG1 (anti-P-selectin, anti-CD62P), was purchased from Pharmingen, San Diego, Calif. Clone W6/32, mouse IgG (anti-HLA-ABC, anti-MHC-I), and clone CR3/43, mouse IgG (anti-HLA-DR, anti-MHC-II), were purchased from Dakopatts (Gynea, N.S.W., Australia). Mouse anti-West Nile virus (WNV) was a kind gift of A. Müllbacher, Division of Cell Biology, John Curtin School of Medical Research, Australian National University, Canberra, New South Wales, Australia.

All antibodies were titrated by flow cytometry (FCM) before use and used at twice the minimum concentration giving saturation labelling.

Virus stocks and cytokines. WNV (Sarafend strain) was grown and cultured as described previously (23) with minor modifications (see below). Semliki Forest virus (SFV) was a kind gift of A. Müllbacher. Both virus stocks used to infect endothelial cells were generated in the same way. Briefly, 4 ml of stock virus was seeded onto washed monolayers of Vero cells cultured in 175-cm² flasks (Nunc, Roskilde, Denmark). Cultures were incubated for 60 min under standard culture conditions (SCC) (i.e., at 37°C in a humidified atmosphere of 5% CO₂ and air), overlaid with 10 ml of Dulbecco's modified Eagle's medium (Commonwealth Serum Laboratories [CSL], Melbourne, Victoria, Australia) supplemented with 10% bovine fetal calf serum (FCS; CSL) and antibiotics, and further incubated for 30 h under SCC. At this point, the supernatant was removed and centrifuged to remove cellular debris, and the clarified supernatant was kept in 1-ml aliquots at -70°C until required. Titters of virus stocks were determined by serial dilutions on Vero cell monolayers as previously described (61).

Human IFN- γ was a gift from Amersham. Units given are those stated by the manufacturer. IFN- γ was titrated on cells to determine the optimum range of concentrations for the maximal induction of MHC and ICAM-1.

Recombinant human IFN- α and IFN- β and matching neutralizing polyclonal antibodies to human IFN- α/β were purchased from Lee Biomolecular Research Laboratories, Inc. Units given are those stated by the manufacturer.

Recombinant human TNF- α , recombinant human IL-1 α and IL-1 β , and recombinant human IL-4 were purchased from Pharmingen. Neutralizing antibodies to human TNF- α and human IL-1 α were purchased from R & D Systems.

Cell culture and infection. HUVEC were isolated as previously described (18). Briefly, human umbilical veins were filled with collagenase solution, incubated at 37°C for 10 min, and flushed with medium 199 (CSL), and cells were collected by centrifugation. Primary HUVEC were seeded in 0.2% gelatin-coated 75-cm² flasks (Nunc) and used for experiments at between 3 and 6 passages. Cells were cultured in medium 199 supplemented with 20% heat-inactivated FCS (CSL), 100 μ g of endothelial cell growth supplement (bovine brain extract) per ml (35), 50 μ g of bovine lung heparin (Sigma) per ml, 10% glutamine, and antibiotics under SCC until confluence. The cells in the monolayer were confirmed to be endothelial in origin by immunoperoxidase staining with monoclonal antibodies to human von Willebrand factor (Dako).

For experiments, HUVEC were reseeded at 0.4×10^6 /well (confluent) in 0.2% gelatin-coated six-well plates (Nunc) with medium 199-heat-inactivated FCS and cultured overnight. The medium was removed from the cells in the plates and replaced with 0.5 ml of WNV diluted in medium 199 (5 PFU of WNV per cell) or with 0.5 ml of uninfected Vero cell supernatant (mock infection), and the mixture was incubated under SCC. After 1 h of incubation, the virus or mock virus was removed, and the culture plates were washed twice with phosphate-buffered saline (PBS). Fresh medium 199-heat-inactivated FCS (2 ml) or medium 199-heat-inactivated FCS with various cytokines or antibodies was then added, and the cells were cultured for between 0.5 and 72 h under SCC. For experiments longer than 48 h, the medium was renewed at 48-h intervals. For experiments to determine WNV infection rates, HUVEC were seeded in six-well plates containing sterile coverslips (0.2% gelatin coated).

Cell labelling for FCM. At the completion of the incubation period, HUVEC cultures were washed twice with ice-cold PBS (2 ml), removed by careful trypsinization, and then washed with PBS before the addition of primary antibody. Cells (0.15×10^6) were pelleted ($463 \times g$, 3 min, 4°C) and resuspended

with 100 μ l of primary antibody at 4°C for 45 min. After incubation, HUVEC were centrifuged through FCS ($463 \times g$, 3 min, 4°C), and the pelleted cells were resuspended in 100 μ l of polyclonal sheep anti-mouse fluorescein isothiocyanate (FITC)-conjugated IgG (Silenus, Hawthorn, Victoria, Australia) for 45 min at 4°C. The cells were then centrifuged through FCS as described above, and the pelleted cells were resuspended in 300 μ l of medium 199 for FCM.

FCM analysis. FCM analysis was performed with a FACScan (Becton Dickinson, Mountain View, Calif.) equipped with an air-cooled argon ion laser set at 488 nm for excitation. Fluorescence was measured at between 515 and 545 nm (FITC). Forward and side scatter measurements were within the same range for all populations, indicating that there were no cell size changes arising from the different treatment protocols used. The FACScan setup was electronically identical for each experiment, and 10^4 events were collected for each sample. Data analysis was performed with the Lysis II program (Becton Dickinson), and the results are presented either in histograms or in line graphs. Data illustrating representative results from individual experiments are presented in histograms as event number versus log₁₀ fluorescence intensity. For representation in line graphs, mean fluorescence was calculated and used as a measure of relative cell surface molecule expression. Data were normalized against the isotype control fluorescence and amalgamated from at least three experiments. Each value shown represents the mean \pm the standard error of the mean population fluorescence averaged over all experiments as an indication of cell surface molecule expression. For statistical comparisons of two groups, an unpaired, two-tailed Student's *t* test was used. A *P* value of <0.05 was considered significant.

Identification of WNV-infected HUVEC. Sterile coverslips were placed in petri dishes prior to seeding of the HUVEC, which were infected with WNV as described above and incubated overnight. The coverslips were removed before trypsinization and fixed in acetone. After being washed, the coverslips were incubated at 4°C for 45 min with 25 μ l of polyclonal mouse anti-WNV antibody (1:20 dilution) or preimmune mouse serum as a control. Cells were then labelled with sheep anti-mouse FITC-conjugated antibody (25 μ l, 1:20 dilution) (Silenus). The coverslips were finally washed with PBS and medium 199 and then mounted on glass slides in DABCO antifade glycerol (19) for fluorescence microscopy. WNV-infected HUVEC were identified by the characteristic, bright perinuclear fluorescence under fluorescence microscopy (42). Hoechst 33342 (Sigma) was used as a counterstain. The percentage of WNV-infected cells was calculated as a ratio of FITC-labelled (infected) cells to Hoechst-labelled nuclei times 100. Ten fields of view, with not less than 100 cells per field, were counted per coverslip as described previously (53).

For assay of WNV infection by FCM analysis, HUVEC were removed by trypsinization and washed as described above and then fixed with 1% paraformaldehyde-0.25% Triton X-100 in PBS for 20 min. Cells were then washed with ice-cold PBS before the addition of anti-WNV primary antibody or preimmune control serum. After being labelled with primary antibody for 45 min, cell pellets were spun through FCS and labelled with FITC-conjugated antibody as described above and then prepared for FCM analysis.

RESULTS

Kinetics of modulation of adhesion molecule expression on HUVEC by WNV infection. Our previous work showed that the flavivirus WNV is able to increase ICAM-1 expression on human embryonic fibroblasts directly (53). This finding has a number of implications for the efficiency of antiviral immunological processes. It was therefore important to study the effect of flavivirus infection on the expression of endothelial cell adhesion molecules, since endothelial cells facilitate the migration of circulating immune cells to local sites of initial virus infection and inflammation.

HUVEC infected at 5 PFU/cell for 24 h on coverslips were fixed and labelled with anti-WNV antibody as described above. Coverslips were then examined by fluorescence microscopy to confirm that WNV was able to infect these cells. By this method, about 48% of HUVEC were found to be detectably infected with WNV (data not shown), similar to the infection levels seen in fibroblasts (53). To corroborate this finding, HUVEC from the same experiment were fixed and labelled with anti-WNV antibody as described above for FCM analysis. As shown in Fig. 1, about 80% of HUVEC were labelled positively with anti-WNV antibody after 24 h of infection, strongly suggesting that the FCM detection method is more sensitive.

HUVEC were infected or mock infected as described above and incubated for 0.5, 1, 2, 4, and 24 h with WNV or SFV at 5 PFU/cell. Figure 2 shows the expression of E-selectin (Fig. 2a),

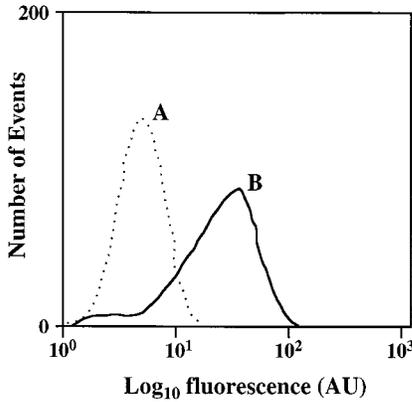


FIG. 1. FCM histograms showing WNV infection in HUVEC. Cells were mock infected (profile A) or WNV infected (profile B) and labelled with anti-WNV antibody and a secondary FITC-conjugated antibody. The ordinate represents the number of fluorescent events, and the abscissa represents \log_{10} fluorescence intensity in arbitrary units (AU).

P-selectin (Fig. 2b), VCAM-1 (Fig. 2c), and ICAM-1 (Fig. 2d) on HUVEC at these times. HUVEC expressed detectable constitutive levels of VCAM-1 and ICAM-1 compared to the isotype antibody controls, but little or no constitutive expression was detectable in the case of E-selectin and P-selectin (data not shown). Significant upregulation of E-selectin on HUVEC by WNV was first detectable ($P = 0.036$) at 0.5 h after WNV infection at 5 PFU/cell. Expression had increased sharply to about two- to threefold ($P = 0.0088$) that in mock-infected controls by 1 h and reached peak levels of about sevenfold ($P = 0.0001$) the mock-infected control fluorescence within 2 h. This was followed by a rapid decrease to about two- to threefold ($P = 0.0003$) the control levels by 4 h. Expression continued to decline and by 24 h had reached levels which were no longer statistically significantly different from mock-infected control levels (Fig. 2a). P-selectin (Fig. 2b) showed a small increase at 4 h in one of the experiments, but the combined data showed no statistical significance at any of the times assayed.

In contrast to that of E-selectin, the expression of VCAM-1 on HUVEC was detectably increased only after 2 h of infection with WNV, when it reached twofold ($P = 0.0063$) the mock-infected (constitutive) control levels (Fig. 2c). This expression further increased to peak levels of about sixfold ($P = 0.0106$) the mock-infected control levels by 4 h but declined to about twofold ($P = 0.0033$) the control levels within 24 h.

Like the expression of VCAM-1, a two- to threefold ($P < 0.0001$) increase in ICAM-1 expression (Fig. 2d) was evident by 2 h in WNV-infected HUVEC, rising to five- to sixfold ($P < 0.0001$) the mock-infected control levels by 4 h. In contrast to VCAM-1 expression, however, this expression was maintained at plateau levels of about fivefold ($P < 0.0012$) the control levels between 4 and 24 h.

To determine whether the induction of adhesion molecules was a feature of neurotropic arbovirus infection of endothelial cells, we infected HUVEC with another plus-strand RNA virus, the alphavirus SFV, since this virus causes leakage of the blood-brain barrier and upregulation of ICAM-1 in vivo in the murine model (55). The percentage of infection of HUVEC with SFV at 5 PFU/cell was similar to that with WNV (data not shown). However, SFV infection of HUVEC caused no statistically significant changes in the expression of E-selectin, P-selectin, VCAM-1, or ICAM-1 at any time at either 5 or 10 PFU/cell (Fig. 2).

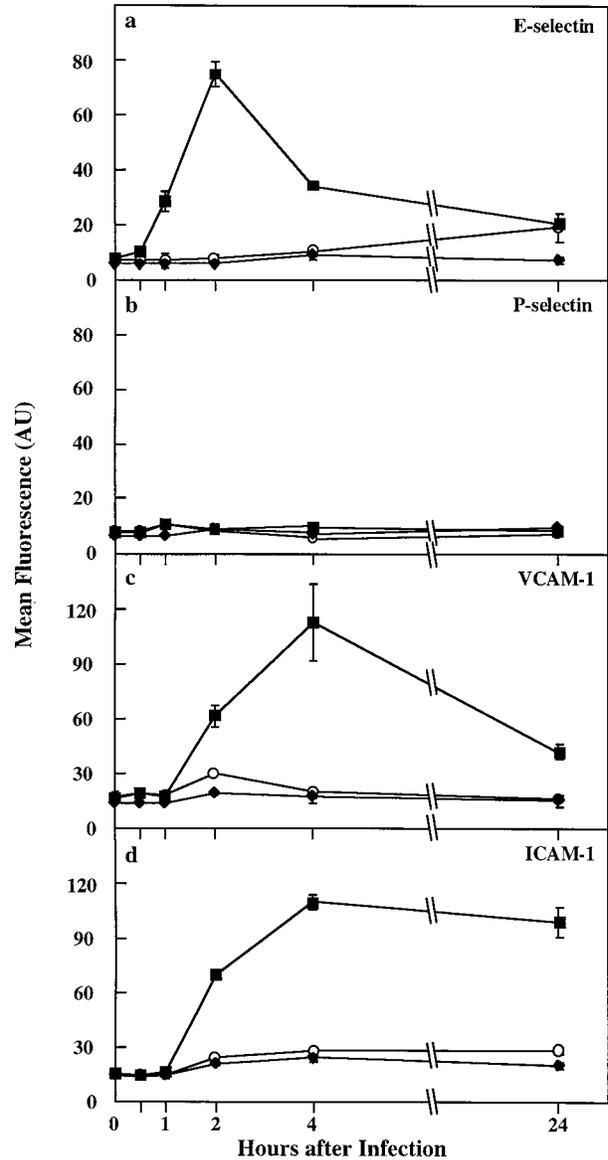


FIG. 2. Kinetics of induction of E-selectin (a), P-selectin (b), VCAM-1 (c), and ICAM-1 (d) expression on WNV-infected (■), SFV-infected (◆), and mock-infected (○) HUVEC. The data represent the means \pm standard errors of the means for four experiments. AU, arbitrary units.

Inactivation of WNV with UV light ($2,400 \mu\text{W}/\text{cm}^2$ for 30 min) destroyed the replicative ability of WNV. If WNV was irradiated immediately prior to infection, this dose completely abrogated the responses described above. UV irradiation that was insufficient to abrogate replication resulted only in the reduction of the upregulation effects on these cell surface molecules, and this effect could be titrated by varying the amount of UV light that the virus received.

Kinetics of E-selectin, P-selectin, VCAM-1, and ICAM-1 expression induced by WNV and immunomodulatory cytokines in combination. Cell-cell recognition in the adaptive immune response is inevitably accompanied by the release of immunological cytokines, usually of either the Th1 or the Th2 spectrum (22). On infected endothelium, such cytokine activity could co- or countermodulate the adhesion molecule upregulation associated with flavivirus infection, influencing leukocyte

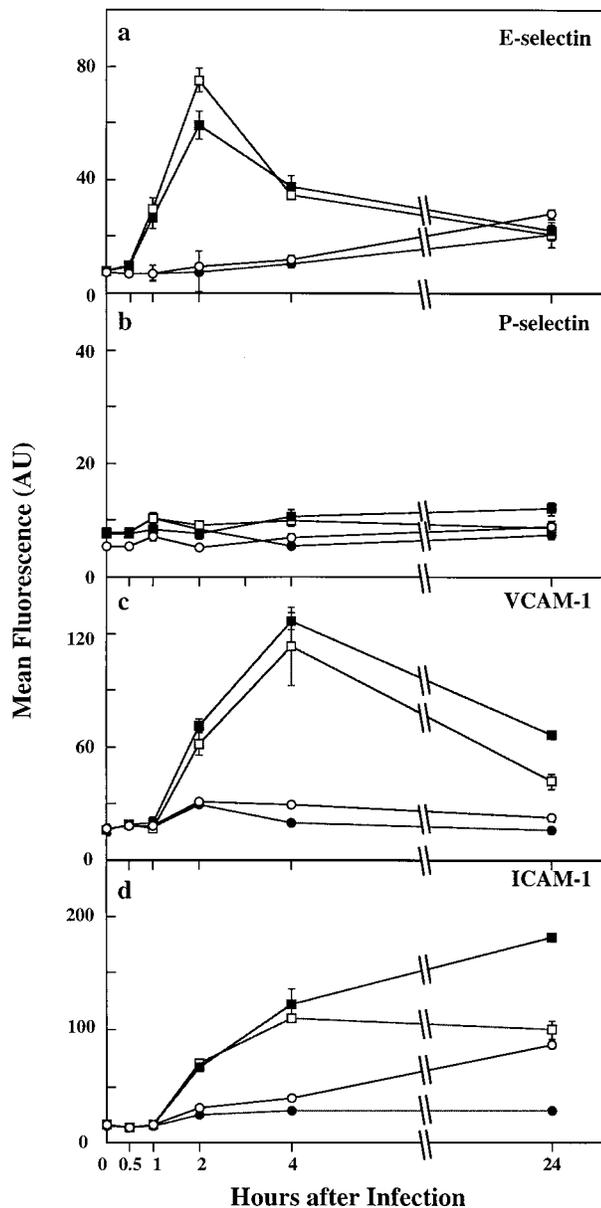


FIG. 3. Kinetics of induction of E-selectin (a), P-selectin (b), VCAM-1 (c), and ICAM-1 (d) expression on mock-treated (●), WNV-infected (□), WNV-infected and IFN- γ -treated (■), and IFN- γ -treated (○) HUVEC. The data represent the means \pm standard errors of the means for four experiments. AU, arbitrary units.

adhesion and thus the generation of the antiviral immune response. Therefore, the effect of cytokines from each spectrum on the expression of adhesion molecules on WNV-infected HUVEC was also investigated.

WNV-infected HUVEC (5 PFU/cell) were incubated in the presence of IFN- γ (100 U/ml), TNF- α (1 ng/ml), IL-1 α (1 ng/ml), or IL-4 (1 ng/ml) and assayed by FCM for the expression of E-selectin, P-selectin, VCAM-1, or ICAM-1 at 2, 4, and 24 h. Control HUVEC were infected with WNV alone, incubated with each cytokine alone at the optimal biologically active concentration, or mock treated and assayed for adhesion molecule expression at the same times.

IFN- γ . Figure 3 shows the results of this experiment with the

classical Th1 cytokine IFN- γ . The combination of IFN- γ and WNV increased E-selectin expression, but the induced levels and kinetics were not significantly different from those obtained with WNV alone at any of the times (Fig. 3a). The combination of IFN- γ and WNV did not alter P-selectin expression at these times (Fig. 3b). In contrast, IFN- γ acted in synergy with WNV infection to upregulate VCAM-1 (Fig. 3c) and ICAM-1 (Fig. 3d) expression by 24 h ($P < 0.0001$ and $P < 0.0001$, respectively) to an extent greater than that observed with either WNV infection or IFN- γ treatment alone, although the kinetics were different. VCAM-1 upregulation followed the kinetics seen with WNV infection, but its decrease at 24 h was delayed by the action of IFN- γ . In contrast, ICAM-1 expression increased in parallel with the kinetics of IFN- γ treatment alone but at about twofold the level on IFN- γ -treated cells. Expression of E-selectin was not significantly altered from mock-treated control levels by incubation with IFN- γ alone, and the same was true of P-selectin. As expected, increased ICAM-1 expression was induced by IFN- γ after 2 h, similar to that seen on fibroblasts (53), this being still greater by 24 h ($P < 0.001$). IFN- γ only weakly increased the expression of VCAM-1.

TNF- α . Results obtained with the combination of TNF- α and WNV infection are shown in Fig. 4. The maximal levels of induced E-selectin expression (Fig. 4a) at 2 h were not different from those caused by TNF- α alone but were slightly higher than those caused by WNV alone, although this difference was not statistically significant. Interestingly, the kinetics of the increases before 4 h followed those of WNV alone, while the kinetics after this time followed those of TNF- α alone. The combination of WNV and TNF- α resulted in an increase in the expression of P-selectin of 3.4-fold ($P < 0.001$) by 4 h, compared to that in mock-treated controls, and this expression increased to 8-fold ($P < 0.0001$) by 24 h and was still increasing when the assay was terminated at this time (Fig. 4b). The increase observed was greater than that caused by TNF- α alone, which caused an increase with slower kinetics. Since WNV did not increase the expression of P-selectin on its own, the results indicate that WNV can nevertheless enhance the induction of this molecule by TNF- α . Like IFN- γ and WNV together, the combination of TNF- α and WNV resulted in significantly higher levels of VCAM-1 (Fig. 4c) and ICAM-1 (Fig. 4d) expression, about 4-fold and 3.5-fold, respectively, by 24 h, compared to those obtained with WNV alone. Increases in VCAM-1 and ICAM-1 expression caused by WNV and TNF- α together by 24 h were about 2-fold and 1.4-fold, respectively, those caused by TNF- α alone.

In TNF- α controls, as expected, TNF- α increased the expression of E-selectin to approximately ninefold the control levels within 2 h of exposure, although notably E-selectin expression was not detectably increased before this time. Thereafter, it gradually decreased to the baseline levels within 24 h (Fig. 4a). There was a small increase in P-selectin expression at 4 h in the TNF- α controls, and this expression was still increasing at 24 h, when it was about threefold the mock-treated control levels (Fig. 4b). VCAM-1 expression was increased from about 2.5-fold ($P = 0.0009$) the constitutive levels by 2 h to about 10-fold ($P = 0.0002$) by 4 h, whereafter it fell to about sixfold ($P = 0.001$) the constitutive levels by 24 h (Fig. 4c). ICAM-1 expression increased throughout this period from about 2.5-fold ($P = 0.0031$) by 2 h to about 4.5-fold ($P < 0.0001$) by 4 h and 9-fold ($P < 0.0001$) by 24 h (Fig. 4d). Increases in VCAM-1 or ICAM-1 expression were not detectable before 2 h.

IL-1 α and IL-1 β . Levels and kinetics of E-selectin induction by IL-1 α treatment and WNV infection together (Fig. 5a) were similar to those obtained with WNV alone, with maximal in-

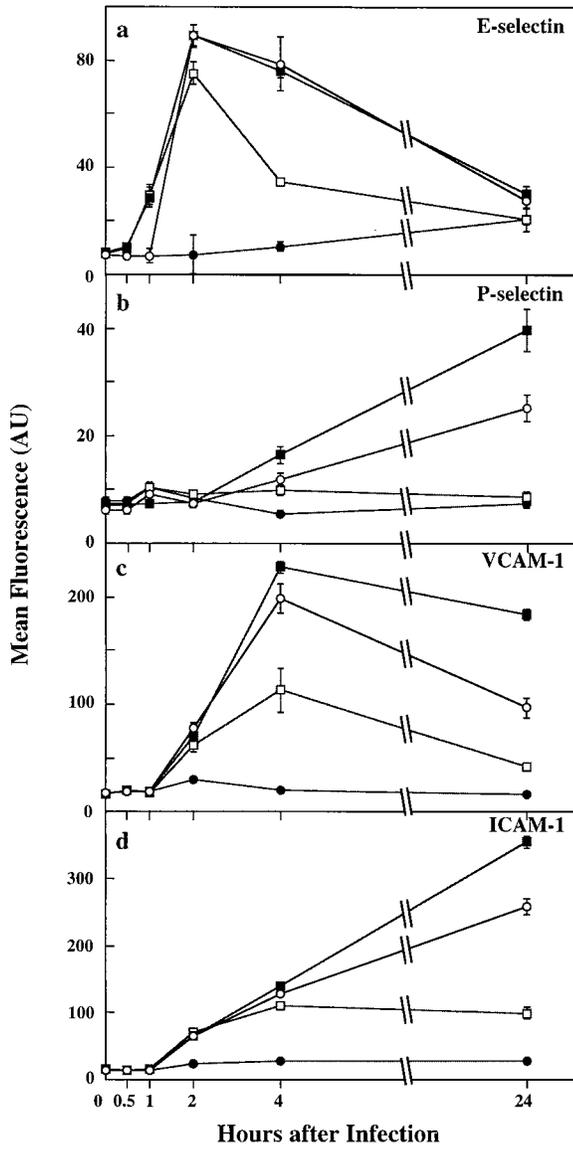


FIG. 4. Kinetics of induction of E-selectin (a), P-selectin (b), VCAM-1 (c), and ICAM-1 (d) expression on mock-treated (●), WNV-infected (□), WNV-infected and TNF- α -treated (■), and TNF- α -treated (○) HUVEC. The data represent the means \pm standard errors of the means for four experiments. AU, arbitrary units.

duction to approximately sevenfold constitutive levels occurring at 2 h and falling to about threefold at 4 h; expression returned to levels not significantly different from control fluorescence by 24 h. These findings were different from the findings obtained with IL-1 α treatment alone, with no detectable induction of E-selectin occurring before 2 h and maximal IL-1 α -induced expression of about fourfold ($P = 0.001$) control levels occurring at 4 h; plateau levels were maintained until 24 h. The effect of WNV and IL-1 α together on P-selectin expression was little different from that of IL-1 α alone (Fig. 5b), although the latter seemed to increase P-selectin expression a little more than the combination of IL-1 α and WNV at 24 h.

No statistically significant differences in VCAM-1 expression were observed at 2 and 4 h with IL-1 α treatment and WNV

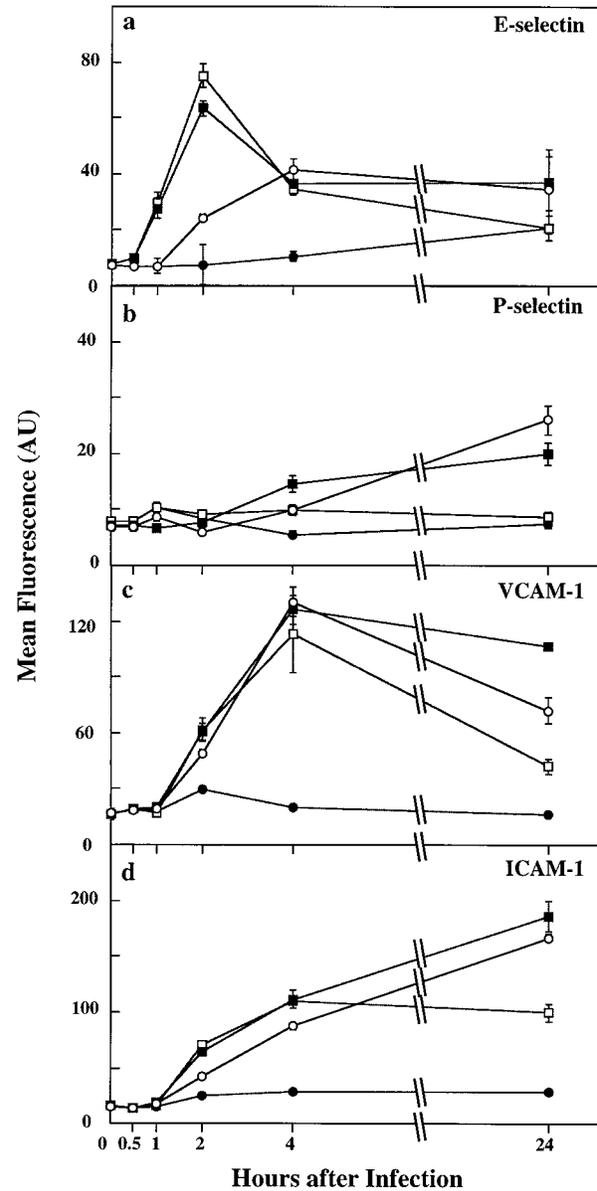


FIG. 5. Kinetics of induction of E-selectin (a), P-selectin (b), VCAM-1 (c), and ICAM-1 (d) expression on mock-treated (●), WNV-infected (□), WNV-infected and IL-1 α -treated (■), and IL-1 α -treated (○) HUVEC. The data represent the means \pm standard errors of the means for four experiments. AU, arbitrary units.

together, relative to either agent alone, all treatments significantly increasing the expression of VCAM-1 at these times (Fig. 5c). However, from 4 to 24 h, IL-1 α and WNV in combination resulted in the maintenance of increased VCAM-1 expression at plateau levels about sixfold constitutive levels. In contrast, with IL-1 α treatment or WNV infection alone, these levels fell over this period at similar rates from about 6.5- or 5.5-fold at 4 h, respectively, to about 4.5-fold ($P = 0.001$) or 2-fold at 24 h, respectively.

With the combination of IL-1 α and WNV, ICAM-1 expression increased to about 2.4-fold ($P < 0.0002$) constitutive levels by 2 h, to 4-fold ($P < 0.001$) by 4 h, and to 6-fold ($P < 0.0001$) by 24 h, with kinetics similar to those of IL-1 α alone (Fig. 5d). Although the higher levels induced at 2 and 4 h by this com-

bination were significantly different ($P = 0.027$ and $P = 0.0428$) from those induced by IL-1 α alone, this difference lost statistical significance by 24 h. This initial difference appeared to have been due to WNV infection, since ICAM-1 induction at these times was identical to that seen with WNV alone. However, by 24 h the combination of IL-1 α and WNV resulted in the induction of ICAM-1 to about twofold that obtained with WNV alone, suggesting a loss of this early viral effect. No induction of VCAM-1 or ICAM-1 by IL-1 α was observed before 2 h.

We also examined the effects of exogenously added IL-1 β , either alone or in combination with WNV, in a manner identical to that used for IL-1 α . The effects on E-selectin, P-selectin, VCAM-1, and ICAM-1 expression under these conditions were very similar to the results shown above for IL-1 α (data not shown).

IL-4. The combination of the classical Th2 cytokine IL-4 and WNV resulted in a clear inhibition at 2 h of the dramatic increase in E-selectin expression caused by WNV infection alone, thus more nearly approximating the kinetics of E-selectin modulation by IL-4 treatment alone than by WNV infection alone. Figure 6a shows that IL-4 alone did not inhibit E-selectin expression, on the contrary causing a small, although statistically insignificant, increase at 4 h, with no effect compared to control fluorescence at the other times. Compared to control fluorescence, IL-4 and WNV together caused a significant increase in P-selectin expression only at 24 h (threefold; $P < 0.001$), but this was greater than the small increase caused by IL-4 alone (Fig. 6b). Therefore, although WNV alone caused no change in P-selectin expression, the addition of IL-4 evidently allowed WNV to enhance the induction of P-selectin expression, similar to the results obtained with TNF- α and WNV in combination.

The combination of IL-4 and WNV resulted in a significant increase in VCAM-1 expression of about 2.6-fold by 2 h, peaking by 4 h to 6-fold constitutive expression (Fig. 6c). The combination increased VCAM-1 expression to a level slightly higher than that caused by WNV infection alone at these times. At 24 h, however, the significant reduction in induced VCAM-1 expression from 6-fold to about 2.2-fold seen with WNV infection alone was delayed by the combination of IL-4 and WNV, with expression falling from 6-fold to about 5-fold constitutive levels.

The combination of IL-4 and WNV resulted in kinetics and levels of ICAM-1 induction indistinguishable from those obtained with WNV infection alone (Fig. 6d). This finding was corroborated by the lack of any induction of ICAM-1 by IL-4 alone in these experiments.

Increased adhesion molecule expression is not due to paracrine effects of WNV-induced IFN- α , IFN- β , TNF- α , or IL-1 production—effect of type 1 IFNs and cytokine-neutralizing antibodies. Although previous work with fibroblasts strongly suggested that cytokines produced by flavivirus-infected cells are not involved in the upregulation of ICAM-1 expression (53), type 1 IFNs (IFN- α and IFN- β) are thought to be responsible for the increases in E-selectin (56) and ICAM-1 expression associated with measles virus infection of glial cells (27). Type 1 IFNs are produced by most, if not all, virus-infected cells and can augment the upregulation of other Ig superfamily molecules, such as MHC-I antigens, in flavivirus infection (23). Therefore, we incubated HUVEC with IFN- α (100 U/ml) or IFN- β (100 U/ml) and assayed for adhesion molecule expression in comparison with that in mock-treated HUVEC at 2, 4, and 24 h. In contrast to our previous findings with fibroblasts (53), we found that at no time did these cytokines induce increased expression of any of the adhesion mol-

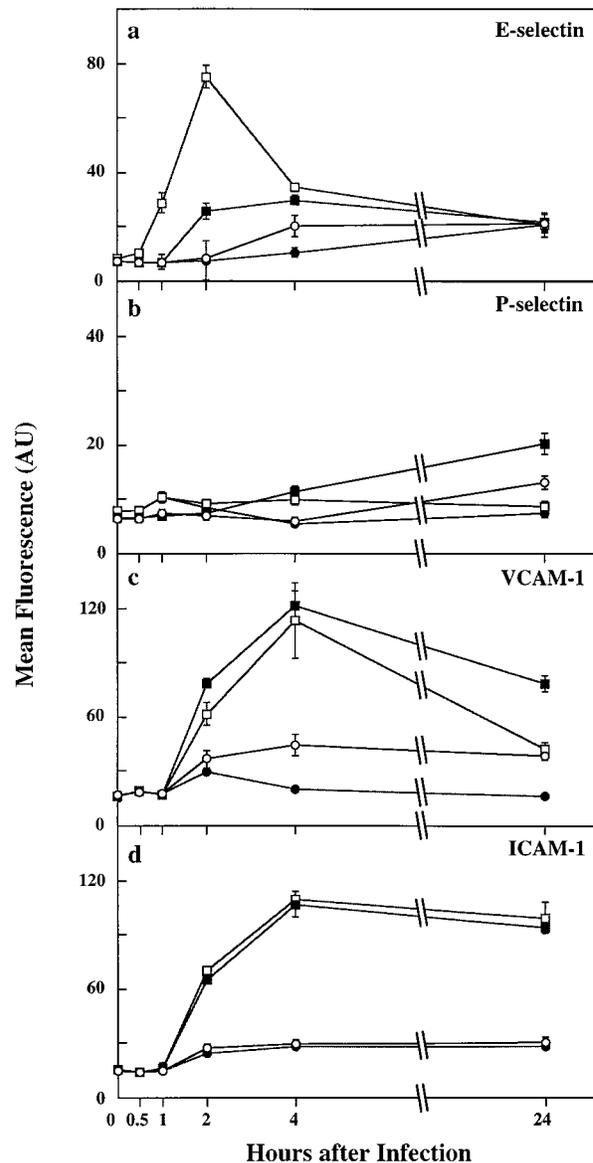


FIG. 6. Kinetics of induction of E-selectin (a), P-selectin (b), VCAM-1 (c), and ICAM-1 (d) expression on mock-treated (●), WNV-infected (□), WNV-infected and IL-4-treated (■), and IL-4-treated (○) HUVEC. The data represent the means \pm standard errors of the means for four experiments. AU, arbitrary units.

ecules tested (Fig. 7). Similarly, the addition of IFN- α or IFN- β to WNV-infected HUVEC did not alter the kinetics or levels of adhesion molecule-inducing responses to infection. In the reverse experiment, neutralizing anti-IFN- α/β antibody (titrated to between 2 and 200 U/ml) (53) did not detectably inhibit the induction of adhesion molecules by WNV infection at any of the times (data not shown). In these experiments, complete inactivation of culture supernatant IFN by antibody was demonstrated as previously described (23).

Endothelial cells also produce TNF- α and IL-1 (38, 60), both of which upregulate different adhesion molecules on endothelial cells to various extents. Therefore, we performed the following experiments to ascertain whether increased adhesion molecule expression in WNV-infected endothelial cell cultures resulted from paracrine effects of WNV-induced TNF- α and

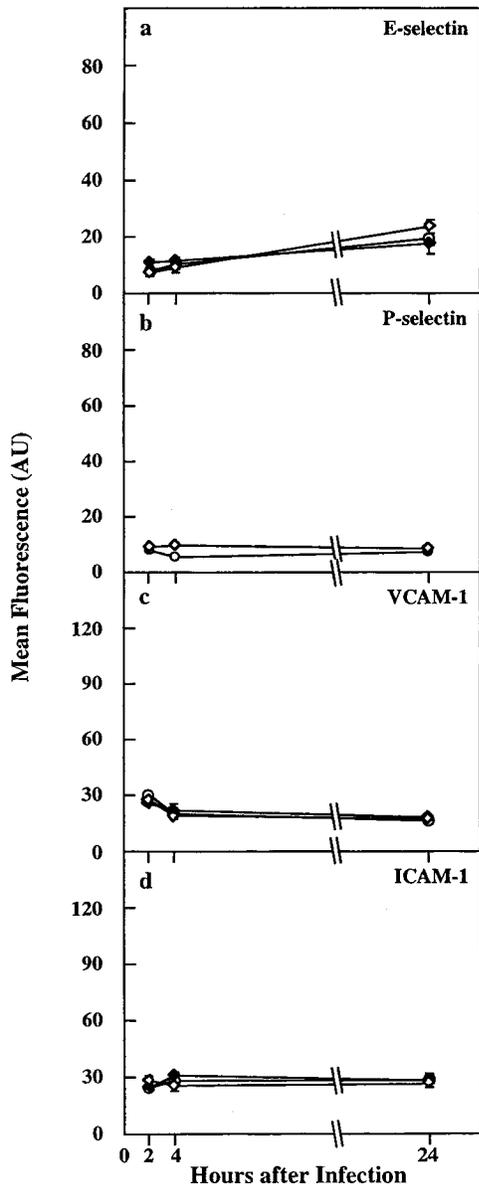


FIG. 7. Expression of E-selectin (a), P-selectin (b), VCAM-1 (c), and ICAM-1 (d) on mock-treated (○), IFN-β-treated (◆), and IFN-α-treated (◇) HUVEC. The data represent the means ± standard errors of the means for four experiments. AU, arbitrary units.

IL-1 release. After WNV (5 PFU/cell) infection for 1 h, HUVEC were washed, the medium was replaced with fresh medium containing neutralizing anti-TNF-α or anti-IL-1α antibodies at concentrations of 0.02 to 2 μg/ml, and the mixture was cultured for 2, 4, or 24 h. Abrogation of the activity of these cytokines was demonstrated as previously described for IFN-α/β (23).

The addition of anti-TNF-α or anti-IL-1α antibodies at maximum concentrations (2 μg/ml) did not abrogate or alter the induction of E-selectin, VCAM-1, or ICAM-1 expression by WNV at 2 h (Fig. 8) or at any other time or dose (data not shown). Since P-selectin was not upregulated by WNV infection, it was not investigated in this assay.

Modulation of MHC on HUVEC by WNV and cytokines. CMV is able to induce MHC-I but not MHC-II expression on

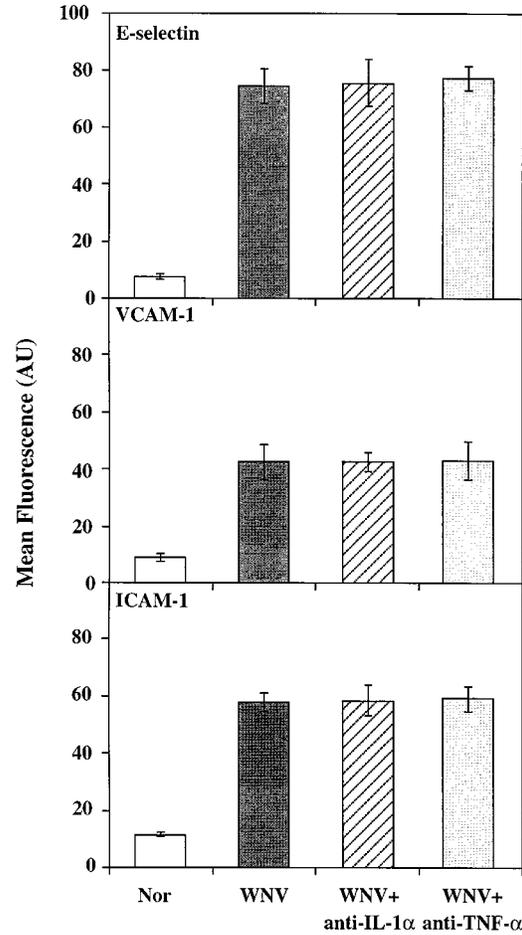


FIG. 8. Expression of E-selectin (a), VCAM-1 (b), and ICAM-1 (c) on mock-infected (Nor), WNV-infected (WNV), WNV-infected and IL-1α antibody-treated (WNV + anti-IL-1α), and WNV-infected and TNF-α antibody-treated (WNV + anti-TNF-α) HUVEC. The data represent the means ± standard errors of the means for four experiments. AU, arbitrary units.

endothelial cells (51, 65), and WNV can upregulate the expression of both MHC-I and MHC-II on some cells (1, 2, 32, 52). Since in concert with the virus-induced modulation of adhesion molecules this fact may have important implications for the immune system recognition of infected cells, we thought it important to determine whether WNV infection could induce increased expression of MHC-I (HLA-ABC) and/or MHC-II (HLA-DR) on HUVEC, either alone or in combination with Th1 or Th2 cytokines.

Readily detectable levels of MHC-I but not MHC-II were found on HUVEC relative to isotype controls (data not shown). As shown in Fig. 9a, MHC-I expression was upregulated by WNV infection (5 PFU/cell) after 24 h ($P < 0.001$) and much more so by 48 h ($P = 0.0001$), with expression remaining at high levels up to 72 h ($P < 0.0001$), when the assay was terminated. Induction of MHC-II expression by WNV (Fig. 9b) occurred only by 72 h, and although statistically significant, the increase was small. There were no detectable changes in MHC-I or MHC-II expression associated with infection by SFV over 24 to 72 h (data not shown). With the addition of anti-IFN-α/β antibody immediately after WNV infection, the increase in MHC-I expression was inhibited by about 30% at 24 h and 40 to 50% at 48 h; by 72 h it was almost

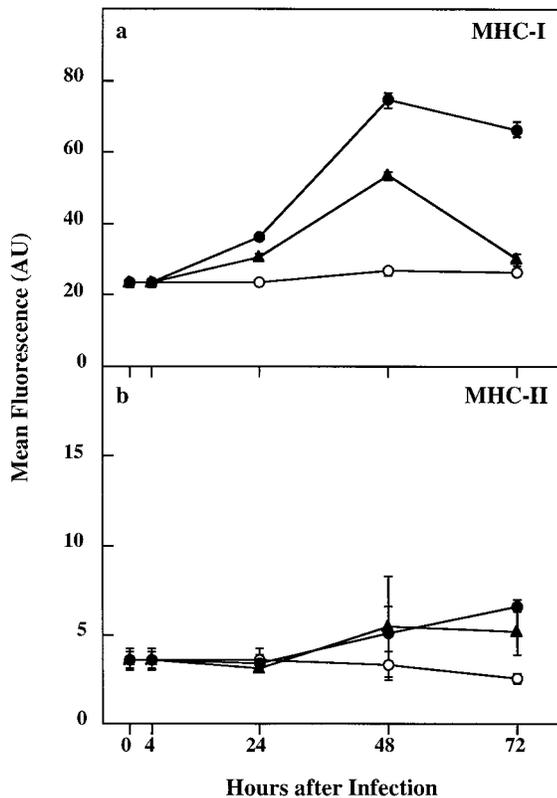


FIG. 9. Kinetics of induction of MHC-I (a) and MHC-II (b) on mock-infected (○), WNV-infected (●), and WNV-infected and IFN- α/β antibody-treated (▲) HUVEC. The data represent the means \pm standard errors of the means for four experiments. AU, arbitrary units.

completely abrogated. No statistically significant changes in WNV-induced MHC-II increases were observed with the addition of anti-IFN- α/β antibody to infected cells. Similarly, the addition of anti-TNF- α and anti-IL-1 α antibodies did not result in modulation of the increases caused by WNV infection over 24 h.

Finally, like the findings with adhesion molecules, the addition of IFN- γ , TNF- α , or IL-1 α to WNV-infected cells significantly increased the expression of MHC-I on these cells over 24 to 72 h to a greater extent than did WNV or any of these respective agents alone. The addition of IFN- β or IL-4 to WNV-infected cells, however, did not increase MHC-I expression more than did WNV alone. The addition of IFN- γ , TNF- α , IL-1 α , and IL-4 to WNV-infected cells over this period resulted in increases in MHC-II expression that were similar to those caused by the cytokines alone but in some cases slightly lower than that caused by the virus alone. Although this finding may be taken to suggest that these cytokines can interfere with the upregulation of MHC-II caused by WNV, the differences were relatively small (data not shown).

DISCUSSION

In this study, we showed that the flavivirus WNV significantly increased the expression of E-selectin, VCAM-1, and ICAM-1 but not P-selectin on HUVEC within 0.5 to 2 h after infection. These increases either preceded or, in a few cases, were contemporaneous with the upregulation effects of all the cytokines tested. In the case of E-selectin, for example, WNV-induced increases preceded those mediated by IFN- γ , TNF- α ,

IL-1 α/β , IL-4, and IFN- α/β by at least 30 min. Although P-selectin induction was not detected, the possibility cannot be excluded that this molecule was mobilized from the Weibel-Palade bodies by WNV infection but decreased to baseline levels before the first time point (58).

In most cases, the WNV-mediated increases in adhesion molecule expression were greater than those mediated by cytokine treatment. However, TNF- α induced all molecules to a greater degree than did WNV, and IL-1 α/β induced higher levels of VCAM-1 and ICAM-1 than did WNV. The speed of induction by WNV and the finding that increases could not be abrogated by neutralizing antibodies to type 1 IFNs, IL-1, or TNF- α suggested that these changes were mediated directly by WNV and not soluble cytokines. Since P-selectin was not upregulated by WNV, it is unlikely that significant amounts of TNF- α were secreted, since P-selectin is readily upregulated by TNF- α (67). In contrast, induction of MHC-I became increasingly susceptible to inhibition by neutralizing anti-IFN- α/β antibody (but not anti-TNF- α or anti-IL-1 antibodies), some 30% reduction occurring at 24 h and increasing to complete abrogation at 72 h. This result is consistent with previous work showing that WNV-induced MHC-I induction results from both direct and type 1 IFN-mediated effects (23) but indicates that these two mechanisms can be separated by different kinetics. The upregulation of MHC-II was the slowest of all the molecules assayed, with small but detectable increases occurring by 72 h and not being abrogated by any neutralizing anticytokine antibodies.

In contrast, no cell surface molecule changes were seen in endothelium infected by the alphavirus SFV. This is also an arthropod-borne, plus-strand RNA virus, which has a similar vertebrate host infection strategy and which is accompanied by local *in vivo* skin responses similar to those seen with flaviviruses (21).

In addition to the direct modulation by WNV, these adhesion molecules were also comodulated in WNV-infected cells by various cytokines. Such comodulation could theoretically occur after viremic endothelial cell infection and leukocyte adhesion *in vivo*. Inflammatory cytokines, including IFN- γ , from the Th1 spectrum of cytokines either enhanced WNV-induced expression, particularly by 24 h, or had no effect in comparison with WNV or cytokine alone. On the other hand, IL-4, a Th2 cytokine, completely abrogated the early E-selectin increases associated with WNV infection while enhancing the effects of WNV on VCAM-1, similar to TNF- α , IL-1, and IFN- γ .

Of further interest was that TNF- α and, to a lesser extent, IL-4 increased P-selectin expression in combination with WNV to a greater degree than did the cytokines alone at 4 and 24 h. Thus, although WNV failed to increase P-selectin expression significantly in its own right, it further enhanced the increased expression mediated by these cytokines.

WNV therefore appears to generate an "inflammatory" intracellular signal, producing a cellular response to the virus similar to that of inflammatory and Th1-derived cytokines. It follows that Th2-derived cytokines, such as IL-4, might, with progression of an inflammatory response from acute to chronic, reduce margination and emigration in some cells (e.g., neutrophils, monocytes, and some lymphocyte subpopulations) but promote them in others. This activity may alter the immunological kinetics of infection and has implications for breaking immunological tolerance (2, 5) and generating or avoiding autoimmunity in susceptible individuals (39).

The mechanism(s) of WNV-mediated upregulation has not been investigated here. However, further enhancement of WNV-induced cell surface molecule expression by some cyto-

kines, especially TNF- α , suggests that the virus operates via a different pathway than these cytokines, since each cytokine was titrated to produce maximal induction. Moreover, the speed of induction was usually faster than with cytokines alone. It is possible that flaviviruses operate directly via more than one pathway to upregulate the same molecules. Flavivirus-induced MHC upregulation is associated with increased transcription (23) and increased peptide availability (40), both necessary but separate processes in MHC-I upregulation. The abrogation of WNV-induced E-selectin upregulation by IL-4 is intriguing and may relate to the interference with the viral induction pathway, since no changes in infection rates were seen in infected cells treated with IL-4 (data not shown).

The adhesion molecules investigated on endothelial cells in this study are variously important in tethering, rolling, firm adhesion, and diapedesis of leukocytes to foci of infection. In the case of MHC-I and MHC-II, they are also critical for immune system recognition of virus-infected cells by T lymphocytes. Flavivirus-mediated upregulation of MHC-I and MHC-II is functional and significantly enhances the recognition of infected cells by virus- or MHC-specific cytotoxic T cells (11, 25, 32).

These findings are of interest, since many flaviviruses, including WNV, cause encephalitis in the presence of significant viremia (41, 66). The endothelial blood-brain barrier normally forms an occlusive seal against macromolecules, including viruses. Thus, encephalitis can probably only occur by passive virus transport across the endothelium, by diapedesis of infected cells into the brain, or by endothelial infection (20). Which mechanism predominates in flavivirus infection *in vivo* is not known, but clearly endothelial cells are readily infected by WNV. It is tempting to speculate that attachment, adhesion, and migration of neutrophils, monocytes, and assorted lymphocyte subpopulations, sequentially associated with the temporal upregulation of the relevant adhesion molecules, might occur in flavivirus-infected endothelium. The cytokine responses of attached leukocytes could further modulate adhesion molecule expression, both on endothelium and on the leukocytes themselves. Infected endothelium, apart from potentially transmitting progeny virus antiluminally directly into the brain, could also infect activated leukocytes which have become avidly attached. Activated cells cross the blood-brain barrier normally (17) and could potentially carry virus with them to infect cells in the brain. Both specific activated lymphocytes and nonspecific inflammatory cells would presumably accumulate in subsequent foci of central nervous system infection (33).

Finally, a further level of complexity to the interaction between virus and host may include the involvement of nitric oxide (NO). WNV induces NO production in monocytes/macrophages associated with adherence and adhesion molecule upregulation (52). Nitric oxide can downregulate leukocyte-endothelium adherence (10) in association with endothelial adhesion molecule downregulation. It is possible therefore, that infected monocytes modulate adhesion to endothelial cells via NO production.

Our present efforts are therefore directed at determining the relative contributions of flavivirus- and cytokine-modulated adhesion molecule expression and NO on endothelial cells to leukocyte attachment and migration *in vivo*.

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