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Cytomegalovirus Inhibits Major Histocompatibility Class II Expression on Infected Endothelial Cells

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Persistent human cytomegalovirus (HCMV) infections are responsible for significant morbidity and mortality in immunocompromised individuals. One mechanism by which HCMV may develop persistence after primary infection is through inhibition of host cell human leukocyte antigen (HLA) class II expression with resultant escape from normal antiviral immune surveillance. Immunofluorescence flow cytometry of human endothelial cell (EC) cultures infected with HCMV AD169 and an EC propagated strain, VHL/E, showed a marked reduction in interferon-γ (IFN-γ)-induced surface expression of HLA-DR. This inhibition did not occur when EC were treated with ultraviolet-inactivated virus and IFN-γ. HCMV, as determined by dual-labeling immunohistochemistry, inhibited induction of surface and cytoplasmic class II antigens specifically in infected cells. HCMV infection also inhibited IFN-γ and tumor necrosis factor-α up-regulation of HLA class I expression. Northern blot analysis of infected, IFN-γ-treated human umbilical vein endothelial cells revealed an absence of class II mRNA. Persistence of HCMV may result in part from its ability to inhibit HLA class II induction in infected cells. (Am J Pathol 1994, 144:683–692)

Infection with human cytomegalovirus (HCMV), a beta herpesvirus with worldwide distribution, is associated with significant mortality in individuals in whom cell-mediated immunity is suppressed. These infections result from either primary HCMV infections or from dissemination of persistent virus.[1]

The mechanisms by which viruses develop persistent infectious states are not completely characterized.[2,3] One mechanism may be avoidance of cell-mediated antiviral immune surveillance as a result of virus-induced reduction in major histocompatibility complex (MHC) antigen expression on infected cells.[4] Both human adenovirus and HCMV have been shown to down-regulate MHC class I expression on infected cells.[5,6] This has also been shown for the murine lymphocytic choriomeningitis virus (MLCMV), for which the lack of class I expression on infected neurons results in resistance to MLCMV-specific T cell-mediated cytolysis.[7]

The role of MHC class II antigen expression in viral persistence is unknown. Although MHC class I molecules appear to represent the principal target for cell-mediated antiviral responses, class II molecules may also function in the response to viral infections.[8] Endogenous viral antigens can be presented in the context of class II molecules and be recognized by class II-restricted cytotoxic T cells.[9–12] $CD4^+$ T cells isolated from HCMV-seropositive individuals proliferate in response to autologous presentation of HCMV antigens,[13] and HLA class II-positive HCMV-infected cells can be specifically lysed by $CD4^+$ T cells.[14] These findings suggest a role for class II in mediation of the normal cellular immune response to HCMV and provide a basis for hypothesizing that viral-induced alterations in class II antigens may contribute to persistence. In the mouse, both murine hepatitis virus and Kirsten murine sarcoma virus have been shown to inhibit the induction of MHC class II expression by interferon-γ (IFN-γ) on infected cells.[15,16]

Endothelial cells (ECs) can be infected by HCMV *in vivo* and *in vitro* and may represent a site of persistence.[17–20] In addition, they possess a diverse array of immunological functions including expression of HLA class II molecules in response to IFN-γ.[21,22] ECs-expressing class II molecules are capable of presenting antigens and can be specifically lysed by cytotoxic $CD4^+$ T lymphocytes.[23] The

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effect of HCMV on EC class II induction is controversial. Van Dorp et al[24] reported that HCMV AD169 infection of HUVEC did not interfere with IFN-γ-mediated induction of HLA class II, whereas Scholz et al[25] reported that HUVEC infected with HCMV strain AD169 reduced IFN-γ up-regulation of class II.

In this study we tested the hypotheses that viable infectious HCMV inhibits the induction of HLA class II induction, that the inhibition occurs specifically on and within infected cells, and that the inhibition results from failure of infected cells to transcribe class II mRNA. This study was facilitated by use of a cytomegalovirus (CMV) strain, VHL/E, that has been serially propagated through multiple passages in human umbilical vein endothelial cells (HUVEC) cultures and exhibits considerably greater endothelial cytopathogenicity than HCMV AD169.[26,27]

Materials and Methods

EC Culture

HUVECs were isolated from vessels by a modification[28] of the techniques of Jaffe et al[29] and Gimbrone et al.[30] Cultures were propagated in endothelial cell growth medium (ECGM), consisting of M-199 (GIBCO, Grand Island, NY) supplemented with 20% fetal bovine serum (Hyclone Laboratories, Logan, UT), 100 μg/ml endothelial cell growth factor (ECGF; prepared according to the methods of Maciag et al[31]), 12 U/ml sodium heparin, and 20 mM HEPES buffer. Adult EC (GVEC) were isolated from gonadal veins using a similar protocol (EH Huang et al, manuscript submitted). Gonadal veins were used because they are attached to cadaveric donor kidneys and possess few collaterals. All growing surfaces for HUVECs and GVECs were pretreated with fibronectin (Upstate Biotechnology, Lake Placid, NY). Cultures of passages 3 to 7 were used in all experiments. All HUVEC and GVEC cultures tested negative for mycoplasma and acholeplasma. Briefly, representative aliquots of all HUVEC and GVEC isolates were passaged twice in antibiotic-free medium and subsequently tested for mycoplasma by in-solution hybridization and ribosomal RNA detection with a ^{3}H-labeled DNA probe homologous to mycoplasma and acholeplasma ribosomal RNA (GEN-PROBE, San Diego, CA).

Culture purity was verified by immunostaining with MAb F8/86 to von Willebrand's factor (Dako Corp., Carpinteria, CA). No contaminating leukocytes were present as determined by immunostaining for leukocyte common antigen (MAbs PD7/26, 2B11; Dako), B cell antigen (MAb L26; Dako), and monocyte-specific antigen (MAb S-HCL-3; Becton Dickinson, San Jose, CA).

CMV Infection of Cultured ECs

ECs were infected with CMV strain VHL/E.[26,27] Briefly, this is a clinical isolate that has been serially propagated through multiple passages in HUVEC cultures and exhibits considerably greater endothelial cytopathogenicity than HCMV AD169. Low passage confluent EC monolayers were inoculated in tissue culture flasks (Corning Glass Works, Corning, NY) for 60 minutes with cryopreserved VHL/E stock virus titers of 0.1 plaque-forming units (PFUs)/cell after which they were washed with phosphate-buffered saline (PBS) and incubated with ECGM at 37 C until they contained numerous large confluent plaques. In some experiments a dispersion method was used to more rapidly and uniformly expand populations of infected cells.[32] Briefly, monolayers were washed twice with PBS, released from the substrate by addition of 0.005% trypsin/0.01% EDTA, and resuspended in fresh ECGM. This preparation was combined with additional similarly suspended cells from uninfected cultures replated at near-confluent density in fresh culture flasks and incubated until >95 of the cells exhibited cytopathic change. HUVECs were also infected with CMV strain AD169 (ATCC). AD169 was propagated in human fibroblast cells (MRC5) using standard methods. Confluent monolayers of HUVEC were centrifuge inoculated with AD169 at a MOI of 5 PFU/cell for 60 minutes at 200 × *g* at which time they were washed and incubated in ECGM for various time periods before treatment with recombinant IFN-γ (Chemicon, Temecula, CA) or recombinant tumor necrosis factor-α (TNF-α) (Genzyme, Boston, MA).

The exact extent of CMV infection was assessed by immunohistochemical staining for either CMV early nuclear protein (ENP) (Dupont, Billerica, MA) or for an epitope common to early and late antigens (MAb CCH2; DAKO). Equivalent ECs were maintained under virus-free conditions and included in all experiments as controls.

Determination of Viability

To verify cell viability, samples of infected (or noninfected) cultures were assayed by vital dye exclusion (trypan blue or propidium iodide). Furthermore, the relative abilities of infected or noninfected cells to spread and attach to plastic substrates (plating efficiency) were compared by plating suspended cells at

known concentrations in fibronectin-treated culture wells. After 18 hours incubation, monolayers were washed to remove nonadherent cells, suspended by trypsinization, and counted by hemacytometer. Plating efficiencies of infected and noninfected HUVECs were determined by dividing the mean number of attached cells (18 hours postplating) by the quantity initially deposited/well. Finally, mitochondrial activity in infected and noninfected monolayers was compared using a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).[33]

The tetrazolium ring of MTT (a pale yellow substrate) is cleaved by dehydrogenase enzymes within active mitochondria to yield a dark blue formazan product that can be monitored colorimetrically, providing an index of cell viability and degree of activation. Infected and noninfected HUVECs were plated in fibronectin-treated 96-well flat-bottom microtiter plates (Linbro, Flow Laboratories Inc., McLean, VA) and incubated 18 hours to allow adherence. Medium was then removed and replaced with 175 μl/well ECGM supplemented with 700 μg/ml MTT (Sigma, St. Louis, MO). Plates were incubated for an additional 4 hours, followed by removal of MTT/ECGM, addition of PBS (120 μl/well), and finally addition of acid-alcohol (0.04 N HCl in isopropanol, 120 μl/well). Optical densities ($OD_{570/630\ mm}$) of wells were determined within 30 minutes of development using a multiwell scanning spectrophotometer (EL 312; Bio-Tek Instruments).

Ultraviolet-Inactivated HCMV (UVHCMV)

Virus was inactivated by exposure to a virucidal ultraviolet (UV) source (G30T8, 30 Watt, Germicidal Lamp, 253.7 nM, Sylvania, Japan) for 20 minutes. Successful inactivation of viable virus was confirmed by the inability of UVHCMV to infect HUVEC as determined by culture morphology and immunostaining for HCMV early nuclear antigen. For determination of the effect of UVHCMV on HLA-DR induction, cells were inoculated with either viable virus at an MOI of 0.248×10^4 PFU/cell or with UVHCMV that had an identical preinactivated MOI for 1 hour, washed three times with PBS, and incubated at 37 C in ECGM until there was extensive cytopathic effect in HCMV-infected cultures. This point averaged between 10 and 15 days postinoculation. Both groups of cultures were then treated with IFN-γ, 200 IU/ml, for 96 hours. To maximize any inhibitory effect of UVHCMV, UVHCMV cultures were incubated with a second UVHCMV inoculum for 1 hour immediately before IFN-γ treatment.

Flow Cytometry

For flow cytometry cells were harvested with 0.005% trypsin/ 0.01% EDTA then washed and incubated with fluorescein-labeled MAb, either L243 (Becton Dickinson), reactive with a nonpolymorphic determinant on HLA-DR, anti-HLA class I heavy chain (Olympus, Lake Success, NY), or with anti-ICAM-1 (anti-CD54, Gen Trak, Inc., Plymouth Meeting, PA). A minimum of 5×10^3 cells were analyzed on an Epics Profile flow cytometer (Coulter, Hialeah, FL) calibrated with 2% fluorescent microbeads (Coulter). The absence of dead cells within the gated region was confirmed by propidium iodide staining. In some experiments the HUVEC gates were modified to encompass only cytomegalic HCMV-infected HUVEC (see *Results*). Nonspecific fluorescence was assessed by staining cells with an irrelevant FITC-labeled IgG2a MAb, L17F12 (Becton Dickinson), for HLA-DR, FITC-labeled IgG2b MAb (Olympus) for HLA class I, or an IgG1 MAb, X40 (Becton Dickinson), for ICAM-1.

Immunohistochemistry

HUVEC cultured in chamber slides (Lab Tek, Nunc Inc., Naperville, IL) or prepared as cytocentrifuge slides were air-dried then fixed in 4 C acetone and incubated with primary MAb for 30 minutes and stained using the labeled avidin D technique in which cells are sequentially incubated with biotinylated horse anti-mouse IgG followed by horseradish peroxidase avidin D complex (Vector Laboratories, Burlingame, CA).[34] The chromogen reaction was developed with 3-amino-9-ethylcarbazole in 3% hydrogen peroxide. For dual labeling, cells were prepared as described, incubated with a MAb to CMV ENP, and stained by the avidin D-alkaline phosphatase method with the fast blue chromogen (Vector Laboratories). Subsequently, unbound biotin was blocked (Vector Laboratories) and the second MAb (HLA-DRα, MAb TAL.1B5; Dako), HLA class II β-chain (mAb DK22; Dako), or HLA-DP (mAb B7/21; Becton Dickinson) was applied and detected using the labeled avidin D technique and the chromogen 3-amino-9-ethylcarbazole. For negative controls, primary antibodies were substituted with irrelevant MAbs of the same murine isotype and concentration.

For quantitation of the percentage of infected and noninfected cells expressing class II, the central 300 cells in each slide chamber or cytocentrifuge preparation were counted twice by standard brightfield microscopy and averaged.

mRNA Analysis

Ten micrograms of total cytoplasmic RNA, isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi[35] (RNAzol, Cinna/Biotecx, Friendswood, TX), were separated on a 1% agarose 0.22 M formaldehyde gel and transferred to nylon membranes (HyBond-N, Amersham Corp., Arlington Heights, IL). Labeling, hybridization, and subsequent washing of the nylon membrane was performed using established protocols.[36] Membranes were hybridized with a random primer-labeled ^{32}P-CTP (Boehringer Mannheim, Indianapolis, IN) cDNA probe to HLA-DRα[37] in hybridization buffer containing 50% formamide, 5× standard saline citrate (SSC), 0.1% PVP 40,000, 0.1% Ficoll 400,000, 0.5% SDS, 0.2% BSA, and 2.5 mM EDTA. After hybridization, membranes were washed once with 2× SSC and 0.1% SDS at room temperature for 15 minutes followed by one wash in 0.2× SSC and 0.1% SDS at 56 C for 30 minutes. Densitometry measurements of the ethidium bromide fluorescence of the 18S and 28S ribosomal RNA bands were used to control for the amount of RNA loaded into different lanes.[38]

Results

To determine the effect of HCMV on baseline and IFN-γ-induced class II expression, HUVEC were infected with HCMV strain VHL/E. As demonstrated by cytomegalic change and immunohistochemical staining for ENP, more than 90% of cultured HUVECs were infected at the time of IFN-γ treatment. Flow cytometry with a MAb reactive with a nonpolymorphic determinant on HLA-DR demonstrated that noninfected, nonstimulated HUVEC did not constitutively express HLA-DR on their surface and that infection of HUVEC with HCMV did not induce its expression (Figure 1). In contrast to IFN-γ-treated noninfected HUVEC cultures, there was a marked reduction in the percentage of HLA-DR-positive cells in HCMV-infected HUVEC cultures subsequently treated with IFN-γ (Figure 1) (Table 1). Similar results were seen with GVECs, which represent adult venous endothelial cells (Table 1). HLA class II inhibition was also seen with infection by a fibroblast attenuated strain, HCMV AD169; however, the percentage of reduction was less dramatic than with VHL/E because AD169 consistently infected less than 20% of HUVECs (data not shown).

To determine whether the failure of HCMV-infected cells to express class II was secondary to reduced viability of infected cells, a number of parameters of HUVEC viability were assessed. Results of propidium iodide and trypan blue dye exclusion assays demonstrated that more than 95% of CMV-infected HUVEC had an ability to exclude dye, a value identical to that of noninfected HUVEC. In addition, infected cells were capable of spreading and attaching to fibronectin-coated plastic culture substrate nearly as well as noninfected EC. Plating efficiency of noninfected cells averaged 75 to 77%, whereas that of infected EC averaged 68 to 70%. Finally, mitochondrial activity in CMV-infected cultures, as indicated by tetrazolium ring reduction (MTT assay), was equal to or greater than that within noninfected cultures plated at

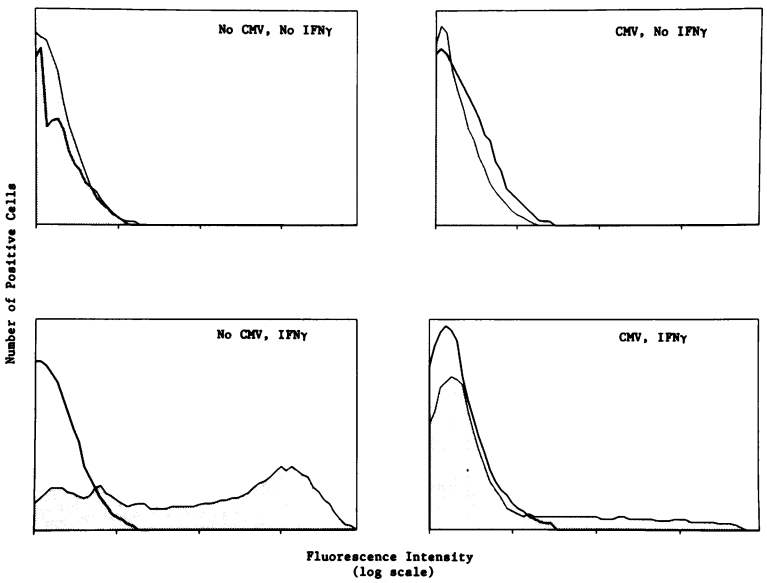

Figure 1. *Effect of HCMV VHL/E infection on HLA-DR expression by endothelial cultures subsequently treated with IFN-γ. Expression of HLA-DR is represented by the shaded area and nonspecific staining using an irrelevant MAb by the nonshaded area under the dark solid line. Cells were treated with 200 IU/ml of IFN-γ for 96 hours when more than 90% of dispersion-infected cells exhibited cytomegalic change* (see Materials and Methods). *The histograms are representative of five experiments with different HUVEC and GVEC isolates.*

Table 1. *Effect of HCMV on IFN-γ Induction of Membrane HLA Class II Expression*

EC Isolates	HLA Class II Expression (Percent "+" EC/Mean Fluorescence*)			
	Baseline	IFN-γ	HCMV	HCMV + IFN-γ
HUVEC 1	0/0	68/117	0/0	9/14
HUVEC 2	0/0	17/24	0/0	1/1
GVEC 1	0/0	78/162	0/0	0/0
GVEC 2	2/5	80/182	1/1	0/0

* Mean fluorescence expressed as mean channel number.

equal cell densities (Figure 2). Collectively, these findings strongly suggest that the loss of IFN-γ responsiveness that we have observed on endothelial infection with CMV cannot be attributed to reduction of viability in infected cultures.

To specifically determine which population of cells, infected or noninfected, failed to express HLA-DR, IFN-γ-treated VHL/E-infected cell cultures were dual immunostained with MAbs specific for HCMV ENP and a monomorphic determinant on the class II β-chain of DR, DP, and DQw1 (Figure 3). Less than 5% of HCMV-infected HUVEC, as represented by CMV ENP-positive nuclei, were class II positive compared with 24% of noninfected cells within the infected cultures. The few HLA class II β-chain-positive infected cells were generally small in size compared with the infected class II β-chain-negative cells that were cytomegalic in appearance (Figure 3). Incubation of infected HUVEC with up to 500 IU/ml of IFN-γ did not alter this pattern of expression. Dual labeling with MAbs to HLA-DRα and HLA-DP showed a similar

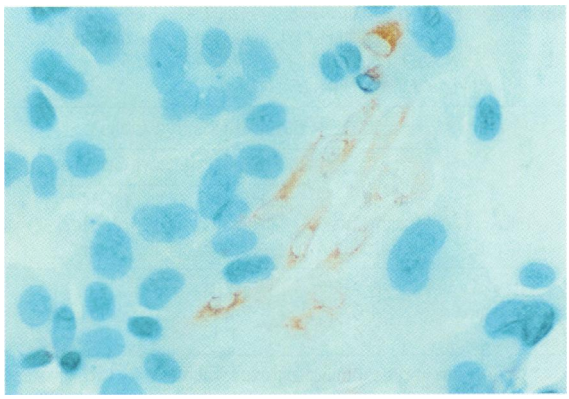

Figure 3. *Expression of HLA class II by HCMV VHL/E-infected and noninfected IFN-γ-treated ECs. ECs were cultured in chamber slides, infected with HCMV VHL/E, and subsequently treated with 200 IU/ml of IFN-γ for 96 hours. Cells were immunohistochemically dual labeled with MAbs specific for CMV ENP (blue stain) and a monomorphic epitope on the HLA class II β-chain (MAb DK22) (red-brown stain). Similar results were observed with two additional HUVEC isolates (original magnification × 100).*

inhibition of IFN-γ-induced class II expression (Table 2). Because the dual labeling immunoperoxidase technique uses acetone fixation, which permeabilizes cell membranes, the staining pattern suggests the cytoplasmic and surface expression was inhibited in infected cells. Reversal of the chromogens or the order of primary antibody application in the dual-labeling technique did not alter this pattern of expression.

Although the results of dual labeling immunohistochemistry suggested that active infection by viable virus was essential for effective inhibition of endothelial class II induction, this finding was further exam-

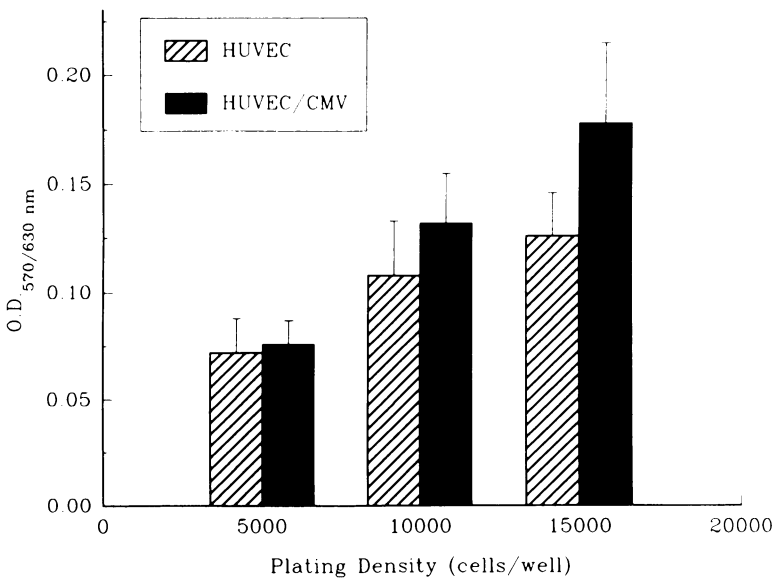

Figure 2. *Mitochondrial activity, as determined by the MTT assay, of CMV-infected HUVEC cultures and noninfected HUVEC cultures at three different culture densities.*

Table 2. *Dual Immunohistochemical Analysis of the Effect of HCMV VHL/E Infection on Induction of HLA-DRα, HLA Class II β-Chain, and HLA-DP by IFN-γ*

HLA Antigen	% HLA Class II-Positive Cells*		
	Noninfected Cultures	HCMV-Infected Cultures	
		HCMV-Negative Cells	HCMV-Infected Cells
DRα	26.3	19.8	3.8
β-chain	34.6	24.1	4.0
DP	3.4	1.1	0.2

* Values are representative of experiments with two different HUVEC isolates.

Table 3. *Effect of UV-Inactivated HCMV on HLA DR Induction*

Treatment	HLA-DR Expression	
	% Positive Cells*	Mean Fluorescent Intensity†
No treatment	0	0
IFN-γ‡	58.6 (2.0)	101.0 (4.4)
HCMV§	0.5 (0.5)	0.1 (0.1)
HCMV + IFN-γ	42.0 (5.6)	52.3 (6.6)
UV-HCMV	0.1 (0.1)	0.2 (0.2)
UV-HCMV + IFN-γ	54.6 (2.7)	99.4 (5.5)

* Mean (SD). Values represent mean of three replicate wells. This experiment is representative of two additional experiments with HUVEC isolates.
† Mean channel number (SD).
‡ 200 IU/ml of IFN-γ for 96 hours.
§ See *Materials and Methods.*

ined by testing the effect of noninfectious virus, ie, UVHCMV, on class II expression. In this experiment HUVECs were inoculated with viable HCMV or UVHCMV and allowed to progress until there were numerous large plaques in actively infected cultures, at which time IFN-γ was added (see *Materials and Methods*). As shown in Table 3, HCMV resulted in a 16.6% reduction in the number of HLA-DR-positive cells that corresponded to the number of cytomegalic HCMV-infected HUVEC within the culture (data not shown). In contrast, the effect of UVHCMV on IFN-γ induction of class II was minimal, both in percentage of positive cells and mean fluorescence intensity. This result supports the hypothesis that complete inhibition of class II seen within a HUVEC requires active viral infection specifically within that cell. Furthermore, it suggests that the inhibitory effect of HCMV is not secondary to virion-mediated physical blockade of IFN-γ binding to its cell membrane receptor.

To determine whether HCMV renders infected ECs refractory to either up-regulation of other inducible surface molecules and/or to other cytokines, the constitutive expression of HLA class I and its up-regulation in response to IFN-γ and TNF-α were examined in infected EC. Treatment of noninfected ECs with either IFN-γ or TNF-α resulted in an increase in the mean surface expression of HLA class I (Table 4). In contrast, HCMV infection resulted in a decrease in the percentage of positive cells and a decrease in mean surface expression. This down-regulation was refractory to either IFN-γ or TNF-α treatment. These results suggest that the inhibitory effect of HCMV infection may not be specific for HLA class II or IFN-γ.

To determine whether any surface molecules are up-regulated by HCMV infection, the expression of ICAM-1 on infected ECs was examined. ICAM-1 has recently been reported to be up-regulated on HCMV-infected fibroblasts.[39] In this experiment cytomegalic cells were specifically selected for analysis by flow cytometry because they were the population of infected cells (as shown in Figure 3) that were resistant to IFN-γ induction of HLA class II. ICAM-1 expression was markedly increased on cytomegalic HCMV-infected HUVEC compared with noninfected cells (Table 5). Cytomegalic cells exhibited higher levels of surface ICAM-1 than noncytomegalic cells, as determined by selective gating on both populations (data not shown). Treatment of noninfected HUVEC with IFN-γ resulted in increased expression of ICAM-1; however, ICAM-1 expression on cytomegalic-infected cells was not increased after identical treatment (Table 5).

To determine whether the lack of inducible HLA class II expression within infected HUVEC represented specific inhibition of HLA class II transcription, HCMV-infected and IFN-γ-treated HUVEC cultures were analyzed by Northern blot analysis for the presence of HLA class II mRNA, using a 1.3-kb cDNA probe to HLA-DRα (Figure 4). Nonstimulated, noninfected HUVECs did not contain detectable HLA-DRα mRNA. HUVECs that had been stimulated for 72 hours with 200 IU/ml of IFN-γ expressed a distinct

Table 4. *Effect of HCMV on HLA Class I Expression and Response to IFN-γ and TNF-α*

Treatment	HLA Class I Expression	
	% Positive Cells*	Mean Fluorescent Intensity†
No treatment	99 (0.3)	156 (23.0)
IFN-γ‡	100 (0.0)	241 (16.9)
TNF-α§	99 (0.2)	225 (13.1)
HCMV¶	22 (17.7)	32 (22.1)
HCMV + INF-γ	31 (26.0)	56 (56.9)
HCMV + TNF-α	24 (20.3)	36 (25.9)

* Mean (SD) of three different EC isolates.
† Mean channel number (SD).
‡ 200 IU/ml of IFN-γ for 96 hours.
§ 20 IU/ml of TNF-α for 24 hours.
¶ See *Materials and Methods.*

Table 5. *Effect of HCMV on ICAM-1 Expression and Response to IFN-γ*

Treatment	ICAM-1 Expression	
	% Positive Cells*	Mean Fluorescent Intensity†
No treatment	9.7 (0.5)	6.0 (0.1)
IFN-γ‡	40.9 (1.0)	23.1 (1.0)
HCMV§	92.0 (0.4)	202.1 (0.8)
HCMV + IFN-γ	91.2 (1.3)	203.2 (3.8)

* Mean (SD). Values represent mean of three replicate wells.
† Mean channel number (SD).
‡ 200 IU/ml of IFN-γ for 96 hours.
§ See *Materials and Methods*.

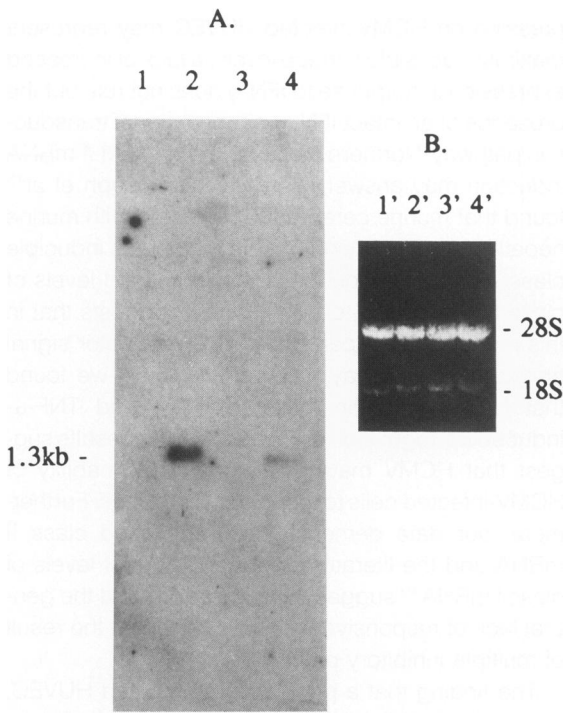

Figure 4. **A:** *Northern blot hybridization with a random primer-labeled ^{32}P-CTP cDNA probe to DRα. Lanes 1 to 4: Nontreated, noninfected HUVEC; IFN-γ-treated, noninfected HUVEC; nontreated, HCMV-infected HUVEC; IFN-γ-treated, HCMV-infected HUVEC, respectively.* **B:** *Ethidium bromide stain showing the relative amounts of RNA; lanes 1 to 4 correspond to lanes 1 to 4 in* **A**.

1.3-kb HLA-DRα transcript. Unstimulated HUVECs infected with VHL/E did not show evidence of HLA-DRα transcription. Infected HUVECs stimulated with 200 IU/ml of IFN-γ for 72 hours showed less than 10% of the 1.3-kb transcript seen with IFN-γ alone, as determined by densitometric analysis. The small amount of HLA-DRα mRNA seen within the induced infected HUVEC most probably originated from the small population of noninfected cells within the infected culture induced by IFN-γ to express HLA class II, as shown in Figure 3.

Discussion

These experiments are the first to convincingly demonstrate that a human virus, specifically CMV, is capable of inhibiting the expression of IFN-γ-inducible HLA class II antigens specifically within infected cells. The reduced class II mRNA levels in IFN-γ-treated infected cultures, as demonstrated by Northern blot analysis, suggests that this phenomenon is secondary to an inhibition of HLA class II transcription. This mechanism of inhibition is clearly different from that demonstrated for HCMV inhibition of HLA class I expression in which class I mRNA is present but an HCMV homologue of the HLA class I heavy chain binds beta-2 microglobulin and inhibits migration of class I molecules to the cell surface.[40]

Inhibition of MHC class II induction has been reported for other microorganisms in nonhuman *in vitro* models. Both murine hepatitis virus and Kirsten murine sarcoma virus block class II transcription in infected cells.[15,16] In contrast to our findings regarding the ineffectiveness of UV-inactivated CMV in inhibiting induction (Table 3), nonviable murine hepatitis virus is as effective as viable virus in inhibiting MHC class II expression.[15] Inhibition of MHC class II induction by IFN-γ is not limited to viruses. Infection of murine macrophages with either *Leishmania donovani* or mycobacteria inhibits class II induction, which in the case of *L. donovani* appears to result from an inhibition of class II transcription.[41–43]

The finding that HCMV inhibits class II induction is at odds with two previous reports. van Dorp et al[24] reported that HCMV infection of HUVEC did not interfere with IFN-γ-mediated induction of HLA class II on HUVEC. It may be that the negative contribution of a small percentage of infected class II-negative cells to a larger population of class II-positive cells was below the limit of detection. This is a common problem with HCMV strain AD169, which minimally infects HUVECs and is the reason we used strain VHL/E, which can infect more than 90% of HUVECs depending on culture conditions. Alternatively, the use by van Dorp et al[24] of HUVECs that had been infected for a shorter period of time before induction may explain the absence of any detectable difference between infected and noninfected cultures. As demonstrated in Figure 3, small newly infected cells are still able to express class II in response to IFN-γ. It is generally only when infected cells begin to show cytomegaly that inhibition occurs. In a second study by Khoury et al[44] HCMV infection of thyroid follicular cells induced HLA-DR, as determined by binding of one of four anti-HLA-DR MAbs. This finding may represent a tissue

specific response to HCMV. Alternatively, it may represent cross-reactivity of the one anti-HLA-DR MAb with an HCMV protein, as has been described in the literature.[45] The results reported herein support and significantly expand the findings of Scholz et al.[25] Specifically, we found that class II inhibition required infectious virions, that the inhibition was most pronounced within infected cells, and that HCMV infection resulted in decreased levels of class II mRNA.

The manner in which HCMV mediates inhibition of HLA class II transcription awaits further elucidation. The ability of HCMV to affect host cell mDNA transcription is well documented. HCMV increases expression of the host genes c-*myc*, c-*fos*, and heat shock protein 70 (hsp70).[46] Increased transcription of hsp70 appears to result from transactivation by a number of HCMV immediate early (IE) gene products including IE1, IE2, UL36–38, and US3.[47] Given these and other reports on the *trans* effects of HCMV immediate early gene products, it is worthwhile to speculate that they may also inhibit IFN-γ-inducible class II transcription. Experiments to test this hypothesis are in progress. Another possibility is that the HCMV genome may contain sequences that exhibit homology with IFN response sequences α and γ, which, as has been suggested for the Kirsten murine sarcoma virus, act in *trans* to down-regulate MHC class II expression.[16] Precedence for homologies between HCMV and host cells exists at the amino acid level. There is a five amino acid sequence homology between HCMV IE-2 and HLA-DRβ that is sufficient to generate MAb cross-reactivity[45] and there is a separate protein with HLA class I heavy chain homology.[48]

HCMV may induce host cell proteins that block IFN-γ-mediated class II expression. Both α and β IFNs, which are induced by virus infections, are capable of inhibiting class II antigen expression on ECs.[49] The data in Table 2, showing that the percentage of HLA class II-positive noninfected HUVECs within infected cultures is less than that of HUVEC within noninfected cultures, suggests the presence of a soluble noninfectious inhibitory substance. However, as shown in Figure 3, the strong expression of class II by uninfected cells adjacent to infected cells argues against a central role for these cytokines in the inhibitory response to IFN-γ. Because the actions of α and β IFNs require binding to a membrane receptor it would be expected that these cytokines, released by infected cells, would bind to immediately proximal noninfected cells and equally inhibit their expression of class II antigens. Experiments in progress using neutralizing antibodies to α and β IFNs suggest that IFNs are not directly involved in the effect of HCMV within the infected cells.[50]

The finding that HCMV-infected cells express increased levels of ICAM-1 suggests that HCMV does not indiscriminately block expression of all surface molecules. The mechanism of increased ICAM-1 expression in HCMV-infected HUVEC is unknown but could represent an increase in transcription, as has been reported for hsp70.[46]

The inhibitory effect of HCMV on HLA class II transcription is unlikely to be secondary to viral blocking of IFN-γ receptor binding, as demonstrated by the UVHCMV studies. However, the presence of an otherwise altered IFN-γ receptor signal transduction pathway can not be ruled out. Because ICAM-1 expression on HCMV-infected HUVEC may represent maximum possible surface levels, a lack of increased expression in response to IFN-γ does not rule out the presence of an intact IFN-γ receptor signal transduction pathway. Northern blot analysis of ICAM-1 mRNA induction may answer this question. Joseph et al[15] found that murine cerebral ECs infected with murine hepatitis virus, although unable to express inducible class II antigens, could express increased levels of class I in response to IFN-γ, which suggests that in this model at least part of the IFN-γ receptor signal transduction pathway is intact. However, we found that HCMV infection inhibited IFN-γ and TNF-α-induced up-regulation of HLA class I. Our results suggest that HCMV may result in general inability of HCMV-infected cells to respond to cytokines. Furthermore, our data demonstrating decreased class II mRNA and the literature reports of normal levels of class I mRNA[40] suggests the possibility that the general lack of responsiveness may represent the result of multiple inhibitory pathways.

The finding that a population of infected HUVEC, always small and noncytomegalic, can express class II in response to IFN-γ, suggests that newly infected cells have insufficient amounts of interfering substance. This is also an important methodological finding in that it demonstrates that the absence of HLA class II antigens in the majority of HCMV-infected cells is not an artifact of the immunohistochemical method used in this study. That is, the dual-labeling method was capable of detecting both HCMV and HLA class II within the same cell.

The ability of HCMV to inhibit HLA class II expression may represent an important mechanism for HCMV persistence, affecting both the afferent and efferent limbs of normal antiviral cellular immune surveillance. HCMV-infected antigen-presenting cells, such as ECs, would be unable to present viral antigens acquired exogenously, and, as recently demonstrated, some endogenous viral antigens. The ability of HCMV to inhibit class II expression would protect

infected ECs, particularly in a locus of IFN-γ-rich cell-mediated inflammation, from immune surveillance mediated by CD4+ T lymphocytes. The findings reported herein, in addition to suggesting a unique molecular mechanism of human viral persistence, also provide another explanation for the generalized immunosuppression associated with HCMV infections. That is, that portion of the afferent immune response dependent on inducible antigen-presenting cell function would be disabled secondary to inhibition of class II expression.

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