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INTERFERON γ POTENTIATES HUMAN CORONAVIRUS OC43 INFECTION OF NEURONAL CELLS BY MODULATION OF HLA CLASS I EXPRESSION

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ABSTRACT

HCN-1A, a human cerebral cortical neuron cell line, was examined for its susceptibility to human coronaviruses. The 229e strain replicated efficiently, but the OC43 strain did not replicate well, if at all. Treatment of the cells with interferonγ at 20U/ml for 48 hr markedly increased the susceptibility of the cells to infection with OC43 virus as shown by a 100-fold increase in secretion of infectious virus over a four day period as compared to untreated controls. The increased susceptibility was shown to be due to membrane expression of HLA class I by receptor-blockade with a monoclonal antibody specific for HLA molecules.

INTRODUCTION

Multiple sclerosis (MS) is characterized by inflammatory demyelination of the central nervous system (CNS) and is believed to be a neurological disease of autoimmune origin for which a viral etiology is suspected (1). The causative virus(es) and its pathogenesis are still undefined, but persistent infection of the CNS with the virus is thought to be involved. The production of subacute and chronic demyelination in rats after infection with mouse hepatitis virus, type 4 (MHV-JHM strain) has led to the hypothesis that this group of viruses may be associated with an MS-like disease of the central nervous system in man (2). Evidence for an association comes from the fact that two coronaviruses, termed SK and SD, were isolated from the brains of two patients with multiple sclerosis (3). Neutralizing antibody was found in the cerebrospinal fluid (CSF) and serum of the patients to the isolated viruses. The

isolates are closely related to murine coronaviruses and human coronavirus OC43 both serologically and by nucleic acid hybridization (4,5). Using the reverse transcriptase-polymerase chain reaction (RT-PCR), murine-related coronavirus RNA and human coronavirus 229e but not OC43 RNA was found in a greater percentage (~35%) of specimens from multiple sclerosis patients than in controls (~20%) (6,7). Also, a sequence of five amino acids predicted from the nucleotide sequence of 229e gene 4, a non-structural gene, is homologous with an immunologically important region of human myelin basic protein (8). However the connection between the coronaviruses and MS is still remote.

In an effort to further explore this hypothesis we evaluated another prediction, namely that a coronavirus subpopulation should be able to replicate in neural cells. Cell culture studies have shown that OC43 virus can cause a persistent infection in a human glioblastoma cell line and that human fetal brain cells are susceptible but without production of infectious virus (9,10).Human coronavirus OC43 recognizes receptors containing O-acetylated sialic acids and utilizes HLA class I as a receptor in several human cell lines (11,12). Since cells of the CNS express very low levels of HLA molecules, we also wanted to evaluate the role of interferonγ (IFNγ) which modulates HLA expression in the pathogenesis of the infecting virus. To our surprise, cortical neuronal cells could support replication of both the 229e and OC43 human coronavirus serotypes. Replication of OC43 virus required enhancement of HLA class I membrane expression induced by IFNγ.

MATERIALS AND METHODS

Cells and Viruses

HCN-1A, a human cerebral cortical neuron cell line, was obtained from ATCC (designation No. CRL 10442) under a material transfer agreement with The Johns Hopkins University, Baltimore, MD. HCN-1A cells were cultured in Dulbecco's MEM containing 4.5g/L glucose with 10% fetal bovine serum (fbs). Human neuroblastoma cell line IMR-32 was also obtained from ATCC. The IMR-32 cell line and MRC-5 human diploid lung cells (Viro-Med, Minnetonka, MN) were grown in Eagle's MEM with 5% fbs.

Stock OC43 and 229e viruses were passaged in MRC-5 cells and plaque purified twice. Cell-free virus was used as inoculum after debris was removed by centrifugation at 1000xg for 10 min. For infectivity assays, virus was inoculated at tenfold dilutions in 0.2ml per 16mm culture well ($2x10^5$ cells per well) onto MRC-5 cells. After 1 hr at 37°C, inoculum was removed and cultures were overlayed with EMEM containing 0.5% agarose. After incubating the infected cells for 4 days at 33°C, the cells were stained with an overlay

medium containing neutral red. Plaques were counted after a further 8 hours of incubation at 33°C and the virus titer was calculated in plaque-forming units (PFU)/ml.

Interferon-gamma treatment prior to infectivity assays

HCN-1A monolayers in 16mm wells were treated for 48 hr with recombinant human interferonγ (r Hu IFNγ) (Upstate Biotechnology, Inc., Lake Placid, NY) at various doses in duplicate. The interferon was removed prior to infecting the cells.

Antibodies

Rabbit anti-OC43 virus antibody was prepared by immunization with gradient purified virus harvested from infected suckling mouse brain tissue, as previously reported (12). Monoclonal antibody (mAb) 4B-6.2 reacting with OC43 nucleocapsid was obtained from Dr. M.J. Buchmeier, The Scripps Research Institute, La Jolla, CA. Guinea pig antiserum to coronavirus 229e was obtained from NIAID, Cat. no. V36-501-558. MAb w6/32, which reacted with assembled human major histocompatibility antigen, HLA, Class I was purchased from Csix Diagnostics, Mequon, WI (13).

Secondary antibodies, goat anti-mouse IgG, anti-guinea pig IgG, and anti-rabbit IgG conjugated to biotin and avidin D fluorescein were obtained from Vector Laboratories, Burlingame, CA.

Immunofluorescence Assays

For detection of viral antigens, the cell monolayers on glass coverslips were washed twice in 0.1M phosphate buffered saline, pH 7.2 (PBS), fixed in ice cold acetone for 10 min, air dried and stored at -20°C. Antiviral antibodies diluted in PBS with 0.1% bovine serum albumin (BSA) were incubated with the fixed cells for 1 hr at 37°C. After five washes with PBS, the secondary antibodies, goat anti-guinea pig or goat anti-mouse biotin were added and incubated for 30 min at 37°C. After five washes avidin D fluorescein was added and incubation was for 30 min at 37°C. After five washes in PBS, the cell monolayers were dipped in water and mounted in Gelvatol (polyvinyl alcohol, 20g, glycerol, 40ml, PBS, 80ml) on glass microscope slides. All preparations were examined with a 10x and 40x objective using a Leitz Dialux 20 photomicroscope equipped with epifluorescence.

To determine HLA class I membrane expression, cell cultures on glass coverslips were incubated with 20U/ml IFNγ for 48 hr, then unfixed cells were stained with mAb w6/32 at 10μg/ml, goat anti-mouse biotin and avidin FITC. Cultures were then fixed with methanol:acetone (50:50,v/v) for 10 min and mounted on glass slides (14).

Receptor blockade assay

Anti HLA class I mAb w6/32 at 1:200 dilution (1μg/ml) was added to the medium and incubated with the cell monolayers for one hour before, during and after infection of the cells (12).

RESULTS

The susceptibility of cultured HCN-1A cells

Cells were infected with 229e and OC43 viruses at a multiplicity of 0.1 PFU per cell and the total virus yield was determined at 24 hr intervals for three days. Table I shows shows that the amount of 229e virus recovered was 0.8 logs greater than that of OC43 virus at 24 hr and increased by 1.3 $logs_{10}$ at 48 hr after infection. In OC43 infected cells, the recovery of virus at low levels for two days never exceeded that of the infecting dose, $2x10^4$ PFU, suggesting that little new virus was synthesized.

When infected cells were examined by immunofluorescence for intracytoplasmic virus antigen at 48 hr post-infection, positive staining was observed in both 229e and OC43 infected cells (Figure 1a and b).

Surface staining for HLA class I on IFNγ-treated HCN-1A cells

It had been reported that treatment of human neuronal cell lines with 10-100U/ml of IFNγ gave a 30-50-fold induction of expression of MHC class I molecules on the cell surface (15). As proof that HLA class I expression could be induced on the HCN-1A cell membranes, the cells were treated with 20U/ml r Hu IFNγ for 48 hr and stained in parallel with unstimulated controls with anti-HLA class I mAb w6/32. Figures 1c and d show that HLA class I molecules were detected on the surface of stimulated neurons. No staining was observed with unstimulated cells.

The effect of IFNγ on OC43 virus replication in HCN-1A cells

Since MHC class I is able to serve as a receptor for OC43 virus, the effect of increasing HLA class I expression on coronavirus OC43 replication in neuronal cells was examined. HCN-1A cells were treated with 20, 2 and 0.2U/ml of r Hu IFNγ for 48 hr at 37°C and then inoculated with OC43 virus at a multiplicity of 10 PFU per cell. The total yield of virus was sampled at 24 hour intervals. As shown in Figure 2, cultures treated with 20U/ml of r Hu IFNγ produced virus steadily over a four-day period reaching a peak of 7.4 $logs_{10}$. The amount of virus produced was more than 1 log_{10} greater than the amount of inoculated virus. Cultures treated with 2 and 0.2U/ml rHuIFNγ showed lower virus yields similar to that obtained with untreated infected cells and virus production decreased after 24 hr.

The effect of antibody to MHC class I on IFNγ-induced potentiation of infection

The ability of mAb w6/32, which binds to conformationally correct HLA class I molecules, to prevent infection of IFNγ-treated neuronal cells by receptor blockade was next examined. Monolayers of HCN-1A cells were treated with 20U/ml of r Hu IFNγ for 48 hr

TABLE I

Coronavirus replication in HCN-1A cells

Virus	titer, pfu/ml, at days post infection[1]		
	1	2	3
229e	$3x10^4$ (0.8)	$1x10^5$ (0)	$4.6x10^2$ (1.1)
OC43	$5.2x10^3$ (0.4)	$5.0x10^3$ (0)	$2.0x10^2$ (1.5)

[1] HCN-1A cell monolayers (2 x 10^5 cells) were infected at a multiplicity of 0.1 in triplicate, washed twice and incubated in EMEM medium with 0.1% fbs. at 24 hr intervals, cells were scraped into the supernatant medium, disrupted by freeze-thaw and titered for infectious virus by plaque assay. The titers are expressed as the average and the standard deviation (in parenthesis).

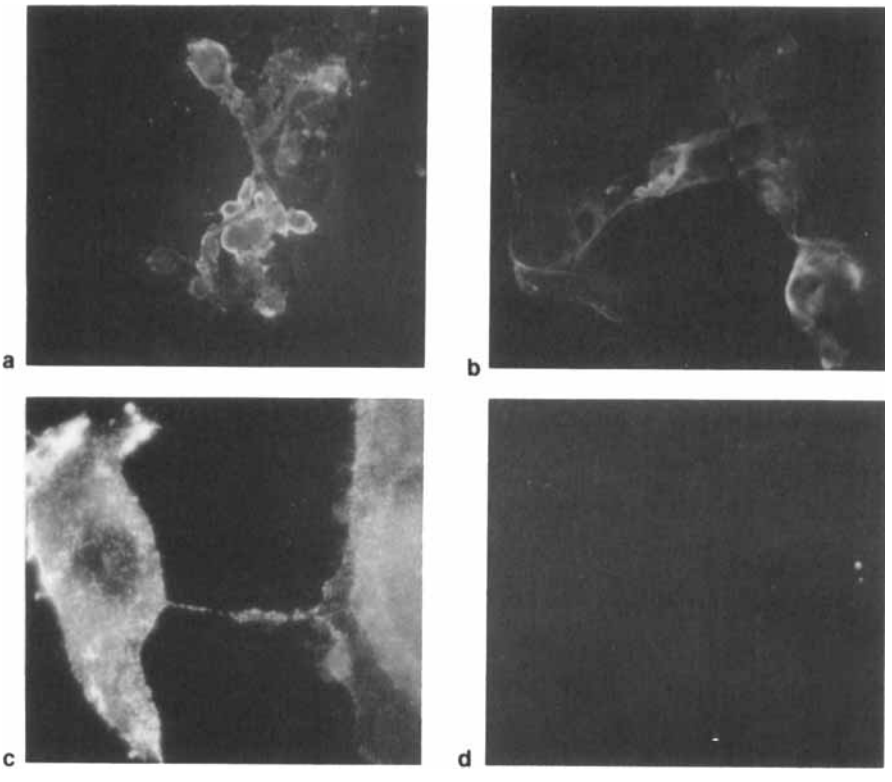

FIGURE 1

Immunofluorescent staining of HCN-1A cells. a. intracytoplasmic OC43 nucleocapsid antigen. b. intracytoplasmic 229e virus antigens. c. membrane expression of HLA class I antigen. d. unstimulated control stained for HLA class I antigen. (x400)

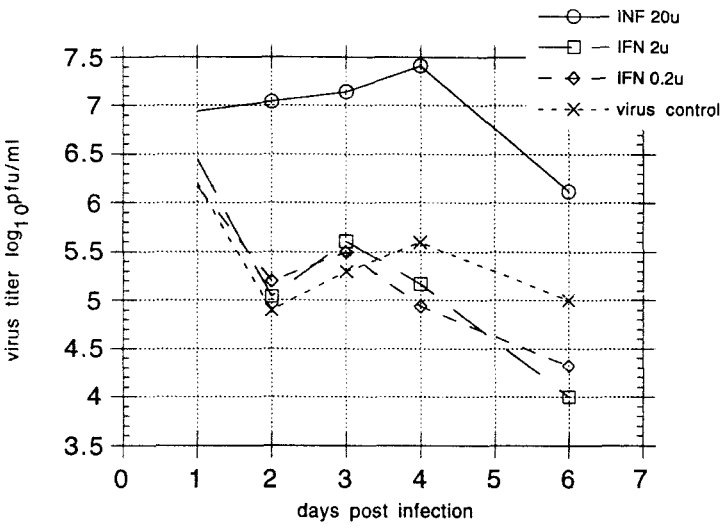

FIGURE 2

Infectious virus synthesis in IFNγ-treated and untreated HCN-1A cells. Confluent monolayers (2 x 10^5 cells) were treated in duplicate with rIFNγ at 20, 2 and 0.2U/ml for 48 hr. Then, along with untreated controls, cells were inoculated with OC43 virus at a multiplicity of 10 for 1 hr at 37°C and washed three times. Low levels (10^2 PFU) of virus were recovered from the supernatant medium after washing. At 24 hr intervals, cells were scraped into the supernatant medium, sonicated for 20 sec and titered for infectious virus by plaque assay. The data represent the average titer from duplicate experiments.

at 37°. One set of cultures was exposed to mAb w6/32 (1μg/ml) for 1 hr prior to and during infection. Cells were infected with OC43 virus at a multiplicity of 1 and incubated at 33°C. Supernatant medium was sampled at 24 hr to determine the titer of released virus. The results of experiments from two neuronal cell lines HCN-1A and IMR-32 are shown in Table 2. The yield of infectious virus in the supernatant medium of IFNγ-treated cells was 2.0 $logs_{10}$ higher than in the infected cells treated with mAb w6/32. Thus the antibody to HLA class I prevented the IFNγ-mediated potentiation of infection.

DISCUSSION

HCN-1A are human cerebral cortical neurons established from grey matter of a patient with unilateral megalencephaly, a disorder associated with continual proliferation of immature neurons (16). The data presented here show that coronaviruses are able to replicate in these cells. Human coronavirus 229e infection of HCN-1A cells is productive

TABLE II

Measurement of protection by antibody to MHC class I on IFNγ mediated potentiation of OC43 virus infection

	Virus yield[1], pfu/ml	
Cells treated with:	HCN-1A	IMR32
virus	$8.8x10^3$ (0.75)	$5x10^2$ (0.5)
virus+IFN[2]	$1.5x10^5$ (0.5)	$4.8x10^4$ (0.7)
virus+IFN+antibody[3]	$1.5x10^3$ (1.0)	$5x10^2$ (1.0)

1 Cells were infected with virus at a multiplicity of 1 in duplicate, washed twice and incubated in EMEM with 0.1% fbs. Supernatant virus yield at 24 hr post infection was determined by plaque assay. Results are expressed as average titer and the standard deviation (in parenthesis).

2 Cell monolayers were treated with 20U/ml IFNγ for 48 hr prior to infection.

3 After treatment as above (2), cells were treated with mAb w6/32, anti HLA class I, at 1ug/ml for one hour before, during and after incubation with virus.

yielding 5 $logs_{10}$ of virus by 48 hours after infection. This result is interesting since neurons express aminopeptidase-N, the receptor for 229e virus at nerve synapses (17). HCN-1A cells are much less susceptible to direct infection by human coronavirus OC43 as shown by the low recovery of infectious virus, 3 $logs_{10}$, and the declining titer over the three day period. However both viruses induced the synthesis of viral antigens which were observed by immunofluorescent staining. Treatment of HCN-1A cells with 20U IFNγ stimulated the cell surface expression of MHC class I molecules and potentiated synthesis of OC43 virus for a period of four days. A similar result was obtained with IMR-32, a neuroblastoma cells line. The potentiation of viral synthesis could not be attributed solely to increases in the amount of inoculated virus (multiplicity of 10) since untreated cells and cells treated with less IFN_γ received the same amount of virus inoculum but produced much less virus over the same period of time. Increasing the amount of inoculated virus could overcome a defect in replication of a mutant virus in cells in culture. This is of particular relevance in herpes simplex virus type 1 (HSV-1) replication in neurons which possess mechanisms to prevent replication and are therefore relatively non-permissive for HSV-1 replication (18).

Previously, OC43 antigen, but not infectious virus production, was detected in primary human fetal neuron cultures (10). In a study of 26 human fetal brain specimens,

IFNγ did not induce HLA class I or class II expression in human fetal neurons (14). Another study of induction of HLA class I on neuronal cell lines demonstrated that expression could be enhanced by IFNγ treatment (15). Stages of development and neoplastic transformation could account for these apparent discrepancies. In this context, it is of interest that phagocytosis of coronavirus particles has been shown to induce HLA class II expression in rat astrocytes (19).

The receptor for OC43 virus requires O-acetylated sialic acid and has been shown to be the major histocompatibility antigen, class I on several human cell lines (11,20). The MHC molecule is a trimolecular complex composed of a polymorphic heavy α chain which is glycosylated, a monomeric light chain called β2 microglobulin and a short peptide of 8 or 9 aa bound in the groove of the heavy chain. The peptide is derived from proteins made in the cell and is necessary to stabilize the α chain-β chain complex. Peptide transporters, coded in the MHC class II region can be induced by IFNγ and function in translocating peptides from the cytoplasm into the endoplasmic reticulum where they are loaded onto MHC class I molecules during assembly. Neurons are deficient in MHC class I expression due to lack of transporters to load peptides onto the MHC molecule and certain neurons, in particular sensory neurons are permanently MHC class I negative (21,22). HCN-1A cells could be insusceptible to OC43 virus for this reason. Testing this hypothesis by stimulating HCN-1A cells with IFNγ prior to infection with OC43 virus resulted in 2 $\log_{10}$ increase in recovery of virus over a four day period, giving positive support for this hypothesis. However, interferons have a two-fold effect on cells; an antiviral effect and an immunoregulatory effect. IFN type II, γ, which is produced by T lymphocytes, primarily functions as a cytokine to increase MHC class I and class II expression although it does have antiviral activity (23). Many viruses have evolved mechanisms for inactivating or degrading the double stranded RNA-dependent protein kinase (PKR) which mediates antiviral activity. Inhibitors include double stranded RNA in high concentration and highly structured RNA species (24). Coronaviral RNA is double stranded during RNA replication and gene 1 assumes a complex pseudoknot structure to allow for ribosomal frameshifting, and so these forms may be PKR inhibitors (25). A related phenomenon has been observed with measles virus isolates passaged in brain tissue. IFN- resistant clones of measles virus are selected by growth of the virus in brain tissue and it has been proposed that this may account for viral persistence in patients with subacute sclerosing panencephalitis (SSPE) (26).

Since the lack of MHC class I expression allows neurons to avoid cytotoxic lymphocyte (CTL) killing, the inducible expression of MHC class I at a certain stage of

neural development may be a mechanism which the virus could use to its advantage to bind to and enter the cell. Persistent infection of human glioblastoma cells by OC43 virus has been reported and co-cultivation of persistently infected glial cells with neurons could spread the infection to neurons (9). Alternatively IFNγ treatment could be affecting the expression of other glycoproteins on the HCN-1A cell surface or molecules within the cell which potentiate viral replication. Further experiments on the binding of HLA and other molecules to OC43 spike glycoprotein are being conducted to define this point.

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