COMPARISON OF THE REPLICATION OF DISTINCT STRAINS OF HUMAN CORONAVIRUS OC43
IN ORGANOTYPIC HUMAN COLON CELLS (Caco-2) AND MOUSE INTESTINE

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Abstract

Three strains of human coronavirus (HCV) OC43 were compared for their ability to cause enteric infections and to induce interferon alpha (IFN α) using the Caco-2 human colon carcinoma cell line which exhibits spontaneous epithelial differentiation in vitro. MRC-5 cell culture grown stocks were prepared from: 1. CV Paris, a strain of OC43 recovered from an outbreak of necrotizing enterocolitis in newborns. 2. CV Mb, a neurotropic strain of OC43 which exhibits strict neuronal specificity in murine neuronal cell cultures. 3. CV Rd, a strain of OC43 which grows to a high titer in human rhabdomyosarcoma (RD) cells. Immunofluorescent staining for nucleocapsid antigen and plaque assay in MRC-5 cells was used to detect viral replication. BG-9 (human foreskin) cells challenged with vesicular stomatitis virus were used to detect IFN α production by human peripheral blood monocytes (PBMC) stimulated by virus infected Caco-2 cells.

Caco-2 cells infected with virus at a multiplicity of infection of 0.5 yielded $10^{4.6}$ and $10^{4.4}$ plaque forming units/ml (pfu/ml) with CV Rd and CV Paris respectively, while CV Mb yielded only 10^3 pfu/ml. Caco-2 cells infected with CV Rd induced 64 IU/ml of IFN α in PBMC while these cells infected with CV Paris induced <2 IU/ml IFN α . In cells infected with CV Mb 4 IU/ml IFN α was detected. The results suggest that a lack of IFN α induction by CV Paris may be an indicator of its enteropathogenic potential.

When newborn Balb/c mice were given 10 μ l of CV Paris orally, viral antigens could be demonstrated in intestinal homogenates inoculated onto RC cells and an antibody titer of 40 at three weeks post infection was shown by immunodot blot. Some virus was recovered from mice given mouse neurotropic CV Mb strain orally but the serum antibody titer was <20. Therefore CV Paris but not CV Mb is enterotropic and induces immunity in mice given the virus orally.

Introduction

Infection with human coronaviruses 229E and OC43 generally produces only mild upper respiratory tract infections which are not life threatening and therefore no special management is required. A few reports have suggested lower respiratory involvement in some cases, especially young children. Routine diagnosis is non-existent in most laboratories (1). Treatment of common colds due to coronaviruses has met with some success. Recombinant IFN α reduced the number of colds in volunteers given IFN α over three to four weeks (2).

Table 1

Human Coronaviruses

<u>Strain</u>	<u>Illness</u>	<u>Serologic Specificity</u>
229E	respiratory	distinct
OC43	respiratory	cross reactive
CV Paris	necrotizing enterocolitis	cross reacts with OC43
HEC A4 HEC C14	necrotizing enterocolitis	distinct
HECV-24 HECV-35	acute gastroenteritis	cross reacts with OC43

Human enteric coronaviruses (HEC) were found in stools from infants with necrotizing enterocolitis (3,4). HEC were also shed in stools from patients with non-bacterial gastroenteritis (5) and an increased incidence of shedding of virus was found to occur from homosexual men with diarrhea who were symptomatic and seropositive for human immunodeficiency virus (6). Two studies of necrotizing enterocolitis yielded cultivable virus. In one study (3), the HEC A14 and HEC C14 virus isolates recovered from the stool were serologically unrelated to OC43 and 229E coronaviruses by immunoblotting. The HEC A14 and HEC C14 isolates produced destructive effects in culture on the brush border of the intestinal epithelium and caused degeneration of the villi. In another case (4), the CV Paris isolate was serologically related to OC43 and bovine coronavirus. In a study of non-cultivable coronaviruses, two isolates HECV-24 and HECV-35 from children with acute gastroenteritis, reacted with antiserum to OC43 by immune electron microscopy. Convalescent sera in the study showed reactivity with a 62kd and a 56kd protein. No antigenic relatedness to OC43 virus was detected by immunoblotting (7,8). Molecular and pathogenic comparison of the human coronaviruses is not available due largely to the difficulty of obtaining isolates of the enteric viruses. A summary of the current strains is shown in Table 1. We have begun to examine what we propose to be several distinct strains of OC43 which could be cultured in MRC-5, human diploid lung fibroblasts. We sought to identify differences in the biological behavior of these strains. Interaction of the viruses with immune system cells was of interest, specifically the stimulation of interferon. Interferon induction by viruses is strictly a property of type 1 (α and β) interferons. However, both interferon gamma ($\text{IFN}\gamma$) and $\text{IFN}\alpha$ are induced in sensitized lymphocytes exposed to viral antigens. In the present study, enteropathology and interferon induction was examined with the possibility in mind that $\text{IFN}\alpha$ may be used to ameliorate the symptoms of chronic diarrhea in individuals with immune system disorders (9,10).

Materials and Methods

Cells: Caco-2 cells, a line of human colonic carcinoma cells, were obtained from the ATCC and maintained in Eagle's MEM with 10% fetal bovine serum (fbs). MRC-5 cells were maintained in Eagle's MEM with 5% fbs.

Virus: CV Paris, a strain of OC43 of probably human origin isolated from the feces of an infant with necrotizing enterocolitis, was obtained from D.A.J. Tyrell, Clinical Disease Center, Harrow, Middlesex, England (4). CV Mb, a

strain of OC43 passaged in suckling mouse brain, was obtained from Cedric Mims, St. Guys Hospital, London, England (11). CV Rd, a strain of OC43 propagated in human rhabdomyosarcoma cells, was obtained from G. Gerna, Pavia, Italy (7). For plaque assay, MRC-5 cell monolayers grown in 24-well tissue culture trays (Costar, Cambridge, MA) were inoculated with 0.2 ml of virus inoculum. After adsorption for 1 h at 37°C, an overlay of 0.5 ml of EMEM with 0.2% fbs and 0.5% agarose was added and the cultures incubated at 33°C. Plaques were visualized on day 4 after addition of a second overlay containing 0.2 mg/ml neutral red. In the 50% plaque reduction assay, 100 plaque forming units (pfu) of virus in 0.2 ml were added to 0.2 ml serial dilutions of heat inactivated antiserum and the mixtures incubated at room temperature for 1 h. Two culture wells were then inoculated with 0.2 ml of each mixture. Plaques were allowed to develop and counted after staining. The titer was defined as the reciprocal of the highest dilution of serum which reduced the number of plaques by 50% of the virus control.

Immunofluorescent staining was performed on acetone fixed cells using a monoclonal antibody (mab) to nucleocapsid (4B-6) obtained from Dr. Michael Buchmeier, Scripps Research Institute, San Diego, CA as previously described (12).

For dot immunoblot assay, microsomal protein (10 µg/3.5 µl) extracted from virus-infected cells was spotted onto a nitrocellulose membrane (0.45 µm pore size) prewet with 0.1 M phosphate buffered saline (PBS) pH 7.2 and allowed to adsorb for 90 min at room temperature in a Biodot Microfiltration apparatus (BioRad Laboratories, Richmond, CA). Following the BioRad procedure, briefly the wells were blocked with 1% BSA, washed, then 100 µl of antibody dilution (1:16,000 for mab 4B-6) was allowed to flow through by gravity for 30-40 min. After three washes, 100 µl of goat anti-mouse IgG, horseradish peroxidase conjugated, at 1:3000 was filtered through the membrane. The washed membrane was placed in substrate solution containing 4-chloro-1-naphthol (Kirkegaard and Perry Laboratories, Gaithersburg, MD) to develop for 10-20 min.

Peripheral blood mononuclear cells were obtained from heparinized human type O donor blood by Ficoll density centrifugation on Lymphoprep (Nyegaarde, Oslo, Norway).

Interferon assays were performed on BG-9 (human foreskin) cells grown in 24-well tissue culture trays by treatment of lightly confluent monolayers overnight with supernatant medium which had been clarified by centrifugation at 1000 xg for 10 min and left at pH 2 for 48 h and returned to neutral pH. The medium was removed and the cultures were challenged with 100 pfu of vesicular stomatitis virus. The cultures were graded for cytopathic effect after 48 h incubation at 37°C and the interferon units (IU) were determined by comparison with an IFNα reference standard (12).

Mice: Pregnant Balb/c mice were obtained from West Seneca Laboratories, West Seneca, NY. All mice tested serologically negative for antibody to OC43 by the plaque reduction assay and the dot immunoblot assay.

Results

Caco-2 cells are highly polarized cells which form microvilli, resembling colonic crypt cells and form domes which are typical transporting epithelial monolayers (13,14,15). These properties favor expression of antigens and infectious virus as it occurs in vivo although the virus inoculum is from cultured cells. Monolayers of Caco-2 cells showing typical dome formation were infected with each strain of OC43 at a multiplicity of infection (m.o.i.) of 0.5. After adsorption of virus for 1 h at 37°C, monolayers were washed twice and maintenance medium with 0.2% fetal bovine serum was added. After 48 h the cells were fixed in acetone and stained by immunofluorescence using monoclonal antibody to nucleocapsid. Figure 1 shows virus antigen in Caco-2 cells infected with CV Paris. Cells containing virus antigen were observed to occur in foci and appeared as aggregates of fused cells which pulled away from the monolayer. Antigen containing cells but no

focal destruction were observed in cells infected with CV Rd and scant antigen was evident in Caco-2 cells infected with CV Mb.

To determine if virus-infected Caco-2 cells will induce interferon in peripheral blood monocytes, cells were infected with each strain at an m.o.i. of 0.5. After 18 h the supernatant medium was removed for virus assay and replaced with medium containing human peripheral blood mononuclear cells (PBMC) at a concentration of 1×10^8 per culture of infected and uninfected cells. After 24 h at 37°C, the supernatants were collected, the PBMC were removed by centrifugation and the supernatant medium was tested for interferon activity. As shown in Table 2, Caco-2 cells infected with CV Paris produced 2.5×10^4 pfu/ml of virus but induced no detectable interferon in PBMC whereas Caco-2 cells infected with CV Rd strain produced 4.5×10^4 pfu/ml and induced 64 IU/ml of interferon in PBMC. The interferon titer was not reduced by pH 2 treatment for 48 h, and was not present in infected Caco-2 cells suggesting that it was IFN α . Only 10^3 pfu/ml of virus was present in the supernatant medium from CV Mb-infected Caco-2 cells and 4 IU/ml of IFN α were induced. No interferon was induced by uninfected Caco-2 cells.

Figure 1. Immunofluorescent staining of Caco-2 cells 48 h after infection with CV Paris at an moi of 0.5. Cells were fixed in acetone, reacted with mab 4B-6 to nucleocapsid, then with biotinylated goat antiserum to mouse IgG and avidin FITC. (magnification: 160x)

Table 2

Interferon α induction by human colon cells
infected with coronaviruses

<u>Virus Strain</u>	<u>Virus Yield (pfu/ml)</u>	<u>IFNα titer (U/ml)</u>
CV Paris	2.5×10^4 ^a	<2 ^b
CV Rd	4.5×10^4	64
CV Mb	3.0×10^3	4

^a At 18 h after infection

^b After 24 h incubation with PBMC (25×10^6 /ml)

In order to compare CV Paris and CV Mb strains for their enteropathogenicity in newborn mice, Balb/c strain mice two to four days old were given 10 μ l orally of a suspension containing 3 to 10 $\times 10^3$ pfu/ml of virus. Intestinal homogenates were prepared from animals taken on day 2, 4 and 7 and virus recovery was sought by plaque assay on MRC-5 cells and by inoculation of RD cell cultures. As shown in Table 3, virus was recovered on day 2 and day 4 from intestinal homogenates of newborn mice given CV Paris and on day 4 from mice given CV Mb. Recovery of CV Paris was confirmed by examination of immunofluorescent-stained RD cells inoculated with the intestinal homogenates. Serum taken three weeks after inoculation was tested for antiviral activity by the plaque reduction assay. Neutralizing antibody to OC43 virus was present at a titer of 80 PRD 50/ml in the serum of mice given CV Paris while no significant neutralization was found in serum from mice given CV Mb. CV Paris antiserum also gave a reaction titer of 40 with the microsol fraction of OC43 virus-infected RD cells in a dot immunoblot assay (Figure 2).

Figure 2

Discussion

We have observed that CV Rd-infected Caco-2 cells induce IFN α in PBMC. The process of induction may be due to viral proteins as has been shown for transmissible gastroenteritis coronavirus (TGEV) (18) or due to the RNA of

Table 3

Enteropathogenicity of human coronaviruses in newborn mice

Virus inoculum ^a	Virus recovery ^b			Antibody titer (PRD ₅₀ /ml)
	d2	d4	d7	
CV Paris	++++	++	--	80
CV Mb	--	+	--	<20
Uninfected	--	--	--	<20

^a 10 μ l (10³ PFU) orally

^b Plaque assay of 10% intestinal homogenate

the infectious virus. The fact that the interferon induction was not a potent response suggests that a mechanism dependent on virus penetration into producer cells is involved. The interferon induced by infected Caco-2 cells was acid stable and therefore considered to be alpha type.

The lack of antibody production and the low level of virus recovered show that CV Mb is not significantly infectious for newborn mice by the oral route. This observation supports the work of Pearson and Mims (15) which showed that this strain of OC43 was neurotropic in newborn mice. The presence of neutralizing antibody and the recovery of virus indicate that CV Paris is infectious for newborn mice by the oral route. To account for the selective tropism of this strain for mouse intestine, a difference in tissue specificity may be postulated. It would be of value to correlate the IFN-inducing capacity of OC43 strains with the development of enteropathogenicity. A protective role of interferon in mice infected with mouse hepatitis type 3 was demonstrated by the use of anti-interferon serum (16). Furthermore, a regulatory role for interferon in coronavirus infections of mice may be inferred from the experience of Weiser and Bang (17) which showed that the supernatant from a mixed lymphocyte reaction (presumably containing interferon and other lymphokines) rendered macrophages from C3H mice susceptible to mouse hepatitis virus. The experiments presented here suggest that CV Paris lacks the ability to induce interferon. Whether the induction depends on viral dose or prior exposure to certain OC43 strains and what is the role of specific lymphocyte subpopulations in induction requires determination. We hypothesize that absence of a vigorous immune response as in individuals with immune disorders, strains of coronaviruses which do not stimulate interferon may have the ability to cause persistent enterocolitis.

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