PERSISTENT INFECTIONS WITH HUMAN PARAINFLUENZAVIRUS TYPE 3 IN TWO CELL LINES

Harold G. Jensen, Alan J. Parkinson, and L. Vernon Scott*
Department of Microbiology & Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

and

Harold G. Muchmore
Departments of Medicine and Microbiology & Immunology, University of Oklahoma Health Sciences Center

Two human cell lines were infected with parainfluenzavirus type 3 (PI3). Growth medium containing PI3 antibody (Ab) was used for establishment and maintenance of persistently infected cultures for 80 subpassages before being replaced with growth medium without PI3 Ab. Persistently infected cells lost infectivity and hemadsorption activities after 20-30 subpassages in growth medium containing specific Ab. Both activities were detected after infected cells were subcultured in growth medium without Ab. PI3 antigen was demonstrated by immunofluorescence in all subpassages. Superinfection studies proved that persistently infected cultures would not support replication of homologous virus. Cultures could be infected with heterologous virus.

INTRODUCTION

Strong evidence that parainfluenzavirus type 1 (PI1) and 3 (PI3) are etiologic agents of midwinter outbreaks of upper respiratory tract illness in a small population of healthy normal human adult subjects totally isolated at South Pole Station, Antarctica has been demonstrated (1, 2). The presence of parainfluenzaviruses in these subjects, long after the accepted incubation period for viral upper respiratory illness at a time when the introduction of new virus into the community is not possible, suggests its persistence in humans. Evidence for PI3 persistence in non-normal humans has been reported (3) in which PI3 was recovered on several successive monthly occasions from adult male patients with chronic pulmonary diseases. PI3 shedding for as long as 115 days has been observed in studies of children with severe immunodeficiency disease (4, 5). These observations demonstrate that human parainfluenzaviruses are capable of persisting in the human upper respiratory tract for prolonged periods, perhaps for life.

An extensive literature exists attesting to the capacity of paramyxoviruses to persist in many cell lines of human (6, 7, 8) and animal origin (8, 9, 10). These descriptive studies of cell cultures persistently infected with paramyxoviruses have indicated that several mechanisms contribute to the establishment and maintenance of persistent infections *in vitro*. These mechanisms include (a) the production of defective virus particles which interfere or modify the replication of mature virus (10), (b) the generation of mutant viral strains with the capacity for noncytolytic replication (11) (c) the infection of specific cell types unable to support the complete replication of virus (12) and (d) possibly provirus formation with insertion of a double-stranded copy of viral RNA into the host cell chromosome (8, 13).

Subsequent to our observations (1, 2) of apparent persisting PI3 infection in normal adults, *in vitro* studies have been undertaken to establish persistent PI3 infections in cell cultures of human upper respiratory tract origin to provide an experimental model for further investigation of factors involved in the initiation and continuation of such infections. This report documents the establishment, preliminary characterization, and maintenance of persistent human PI3 infections in human larynx epidermoid (HEp-2) and human esophageal epithelial (MINN EE) cells.

*To whom inquiries should be addressed.

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MATERIALS AND METHODS

Cell Cultures

Human esophageal epithelial (MINN EE), American Type Culture Collection (ATCC) CCL-4, and human larynx epidermoid (HEp-2), ATCC CCL-23 cells were used for establishing persistent infections. Cells were grown and maintained in Eagle minimum essential medium (MEM) containing 100 units of penicillin and 100 μg of streptomycin per m*l* and supplemented with either 10% newborn bovine serum (NBS) or 10% fetal bovine serum (FBS) (GIBCO).

Sera Used for Cell Culture

By a standard plaque reduction neutralization assay method (14), specific Ab to PI3 was found in the NBS used to supplement MEM during the establishment of persistent infections. This serum had been shown previously to cause a 100% PI3 plaque reduction at a dilution of 1:10 and an 80% plaque reduction at an 1:80 dilution of both NBS and IgG isolated from NBS. FBS was checked also for specific Ab by this method and found not to cause neutralization of infectivity of Ab.

Viruses

PI3, ATCC strain C243, was used in the establishment of persistently infected cell cultures. Herpes simplex virus, type 1 (HSV-1), ATCC strain F (1), was used in superinfection studies. Both viruses were propagated in HEp-2 cells in MEM supplemented with FBS. The infected supernatant fluids to be used for experimentation were harvested after 4 days of incubation at 37 C.

Establishment and Maintenance of Persistent Infections

Monolayers of HEp-2 and MINN EE cells were infected with 10^6 PFU of PI3. After adsorption for 1 hr at 37 C, the monolayers were washed twice with phosphate-buffered saline (PBS). MEM supplemented with 10% (10 m*l* NBS Plus 90 m*l* MEM) NBS containing PI3 antibody was added and the cultures were incubated at 37 C. After 4 days, cells that were not destroyed by lytic infection were washed in PBS and were allowed to form monolayers in fresh medium containing NBS. Monolayers formed from each of the infected cell cultures within two weeks. Both infected and uninfected control cultures of each cell type were subcultured when confluent monolayers were formed within 3 to 4 days. Samples of culture fluid and cells of each subculture were frozen at -70 C for viral assays. After approximately 70 subpassages, FBS was substituted for the NBS in the growth medium. The fetal bovine serum had been shown to contain no specific PI3 Ab.

Detection of Viral Activity in Persistently Infected Cells

To determine if the cell cultures were persistently infected with PI3, four procedures were applied.

Viral Infectivity: The amount of infectious virus from culture fluids of PI3 persistently infected cells was determined by a plaque assay in HEp-2 cells as described by Frickey (15) with minor modifications. Adsorption of virus (0.1 m*l*/plate) was carried out for 1 hr at 37 C in a 5% CO_2 incubator in 35-mm petri dishes. The agar overlay medium, consisting of one part of double-strength MEM and one part of 1.2% Ion #2 agar, was also supplemented with penicillin (100 units/m*l*), streptomycin (100 μg/m*l*) and 2% FBS. After 48-72 hr incubation, 2 m*l* of phosphate buffer containing 10% formalin was added to each dish and was incubated at room temperature for 1 hr. The overlay was then gently removed and discarded and 2 m*l* of a 1:10 dilution of Giemsa stain was added. After staining for 6-8 hr, the Giemsa solution was discarded and the monolayers were washed in tap water and dried before counting. All assays were done in duplicate.

Hemadsorption: The ability of erythrocytes to adsorb to PI3 persistently infected cells was determined microscopically by standard procedures (16) using 0.5% guinea pig erythrocytes at 5 C for 30 min.

Immunofluorescence: Intracellular viral antigen in persistently infected cells that were grown on coverslips was detected by the direct fluorescent antibody technique using fluorescein-conjugated rabbit antiparainfluenzavirus type 3 antiserum (Flow Laboratories). The coverslip monolayers were fixed in acetone, washed 3 times in PBS and stained for 30 min at 37 C with a 1:10 dilution of conjugated antiserum. The stained monolayers were washed in PBS 3 times for 30 min each time, mounted

in a drop of PBS on a microscope slide and examined microscopically for specific fluorescence.

Control experiments using uninfected cells grown in medium supplemented with NBS or FBS were also done. No specific fluorescence was observed. Standard blocking experiments using known immune serum were done to show specificity of the fluorescein-labeled parainfluenzavirus type 3 Ab.

Superinfection: Persistently infected and uninfected (control) cultures were infected with 10^3 PFU of PI3 and were allowed to adsorb for 1 hr at 37 C. The cultures were then washed twice with PBS, MEM with 2% fetal bovine serum was added, and the cultures were incubated for 96 hr at 37 C. The presence of infectious virus was determined by plaque assay in HEp-2 cells. Separate monolayers of persistently infected and control cultures were infected with 100 $TCID_{50}$ of HSV-1 and treated in a manner similar to the PI3-infected cultures just described. However, infectious titers were determined as $TCID_{50}$ in HEp-2 cells grown in 96-well microtiter plates rather than in plastic petri dishes. The $TCID_{50}$ was calculated as described by Reed and Muench (17).

RESULTS

Demonstration Of Virus In Persistently Infected Cell Cultures

Three methods were used to detect and monitor the presence of virus in the persistently infected cell cultures. Infectious PI3 was detected by the plaque assay, viral hemagglutinin was demonstrated by hemadsorption, and intracellular viral antigen was shown by immunofluorescence. Hereafter, when the name of the cell line is followed immediately with PI3, it denotes persistent infection, e.g., HEp-2/PI3.

HEp-2/PI3: A gradual decrease in the amount of infectious PI3 and in hemadsorption activity in HEp-2/PI3 was noted through subpassage 20. After that time neither infectious PI3 nor hemadsorption activity could be detected (Table 1A). Viral antigent was detected by immunofluorescence throughout all subpassages.

Parallel HEp-2/PI3 cultures with MEM plus FBS and MEM plus NBS were started at subpassage 72 (Table 1B). Hemadsorption activity was immediately observed and a low level of infectious PI3 was detected after three subpassages (number 75). At subpassage 90, infectious PI3 had increased over 100-fold and approximately 50% of

TABLE 1. *Detection of persistent parainfluenzavirus type 3 in HEp-2 and MINN EE cells.*

Subpassage Number	Viral Assay: Infectivity[b] HEp-2	Infectivity[b] MINN EE	Hemadsorption[c] HEp-2	Hemadsorption[c] MINN EE	Immunofluorescence[c] HEp-2	Immunofluorescence[c] MINN EE
A. Eagle MEM + NBS[a]						
1	7.0×10^4	2.0×10^5	+++	++	+++	++
10	3.5×10^5	3.0×10^3	+++	++	++	++
20	2.2×10^1	1.0×10^1	+	++	++	++
30	0	1.0×10^1	−	++	++	++
31-80	0	0	−	−	++	++
B. Eagle MEM + FBS[a]						
72	0	0	+	−	++	++
75	3.0×10^1	0	+	+	++	++
77	6.0×10^1	3.0×10^1	++	++	++	++
85	2.0×10^3	1.1×10^3	++	++	++	++
90	5.0×10^3	1.0×10^3	++	++	++	++
95-130	$2\text{-}8 \times 10^4$	$1\text{-}3.5 \times 10^3$	++	++	+++	++

a. NBS, Newborn bovine serum
FBS, Fetal bovine serum
b. Expressed as PFU/0.1 ml.
c. + less than 40% of cells were positive.
++ 40-80% of cells were positive
+++ more than 80% of cells were positive.

the cells adsorbed erythrocytes. The immunofluorescence noted in HEp-2/PI3 was similar in amount to that observed with HEp-2/PI3 cells grown in MEM plus NBS.

MINN EE/PI3: As with HEp-2/PI3 cells, only a small amount of virus was observed in MINN EE/PI3 cells between subpassage 10 and 29 by plaque assay procedures (Table 1A). After subpassage 30, infectious virus was no longer found, but the amount of hemadsorption activity remained constant until subpassage 31, when it could no longer be detected. The number of fluorescing cells was less with the MINN EE/PI3 cells than with the HEp-2/PI3 cells. Specific fluorescence was observed after infectious PI3 and hemadsorption activity was no longer detectable. After 2 subpassages (number 77) in MEM plus FBS, infectious virus was again produced by MINN EE/PI3 cells (Table 1B) and hemadsorption was demonstrated after the first subpassage (number 75). The amount and intensity of specific fluorescence was comparable in cultures containing NBS and those containing FBS.

Superinfection of Persistently Infected Cultures

There was no significant difference in the amount of infectious PI3 recovered from the uninfected persistently infected cell cultures and that from the persistently infected cultures superinfected with PI3 (Table 2). Normal persistently infected cell cultures in MEM plus 2% FBS served as controls to show the amount of virus produced without being superinfected. There was at least a 98% reduction of PI3 in each of the superinfected cell cultures when compared to the virus recovered from the PI3 infected control cells. Both the persistently infected and control cells of each cell line were capable of supporting the growth and replication of HSV-1.

DISCUSSION

Persistent infections of human PI3 were established by serial undiluted passages of virus in HEp-2 and MINN EE cell cultures. Initial cultures were maintained in a medium supplemented with 10% NBS which contained PI3-specific neutralizing Ab, presumably of bovine maternal origin. Although these cultures shed infectious virus during early subpassages, this property together with the hemadsorbing activity of infected cells was gradually lost (Table 1). Despite the neutralization of infectious extracellular virus and hemadsorption, intracellular virus-specific antigens continued to be detected by immunofluorescence.

The induction of a non-yielder state by the addition of Ab to cell cultures persistently infected with other paramyxoviruses has been described. In most cases, non-yielder cultures still contain viral antigens (18). However, Cole and Hetrick (6) reported a gradual loss of bovine PI3 in persistently infected human conjunctival cells through 57 subpassages. Rustigian (19) showed that subculturing measles virus in HeLa cells in the presence of Ab resulted in a cell line which did not release infectious virus. In contrast, the removal of Ab from the persistently infected cell lines in our study and the substitution of NBS with FBS resulted in the gradual reappearance of infectious virus and hemadsorption activity, although the level of PI3 released from these cultures remained at levels from 10-1000 times below the initial infection yields. This reduction in infectious virus

TABLE 2. *Superinfection of persistently infected cell lines with standard PI3 and HSV-1 virus.*

Culture type	PI3 yield per persistently infected culture	Virus Yield	
		PI3[a]	HSV-1[b]
HEp-2	0	3.0×10^{5}	$10^{5.5}$
HEp-2/PI3	1.0×10^{3}	9.5×10^{1}	$10^{5.5}$
MINN EE	0	2.0×10^{4}	$10^{4.5}$
MINN EE/P13	1.3×10^{2}	1.4×10^{2}	$10^{4.3}$

a. PFU/0.1 m*l.*
b. $TCID_{50}$/0.1 m*l.*

in persistently infected cells may reflect either fewer cells being infected and producing infectious virus, or an overall decrease in the rate of viral replication. Experiments to differentiate between these two possibilities using cloned persistently infected cell cultures are currently being conducted in our laboratory.

The mechanisms of establishment of PI3 persistence in HEp-2 and MINN EE cells are unknown. In preliminary studies, the role of temperature-sensitive virus has not been implicated and the role of defective virus particles, suggested in the work of Rustigian (19), has yet to be investigated. It appears unlikely that the presence of virus-specific Ab in the medium played a significant role in the establishment of persistence in these cells, since persistence with PI3 and other paramyxoviruses in a variety of cell types has been established in the absence of virus-specific Ab (6, 9, 10, 19). Hodes, et al. (9) demonstrated the presence of a virus product which inhibited syncytium formation in PI3-infected Vero cells, and they suggested that this inhibitor contributed to the establishment of persistence in these cells. It is possible that this product may also contribute to the establishment of the persistent state in other cell types.

The reappearance of infectious virus after 75-77 subpassages in medium containing virus-specific antibody suggests the maintenance of persistence by transfer of viral genetic material during cell division or a lateral transfer of virus to adjacent susceptible cells. Zhdanov (13) has suggested that reverse transcriptase and integration of RNA-containing virus genomes into the DNA of persistently infected cells, mediated either by cellular reverse transcriptase or by reverse transcriptase from endogenous virus, may play a role in persistent infections with some RNA-containing viruses.

The establishment of MINN EE/PI3 and HEp-2/PI3 demonstrates the potential capacity of PI3 to initiate and maintain a persistent state in cells of the human upper respiratory mucosa, supporting the *in vivo* observations of prolonged PI3 shedding (1, 3, 4), and provides an experimental model for further investigation of factors and mechanisms involved in the initiation, maintenance and reactivation of persistent PI3 infections in humans.

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