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Original Article

Characterization of various strains of influenza B virus in a neuroblastoma and a glioblastoma cell line

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Background and Purpose: Although influenza B virus has been reported to be involved in central nervous system infection, little is known about the infectivity of the virus. We evaluated the ability of several strains of influenza B virus to grow in 2 nerve cell culture systems.

Methods: Five isolates of influenza B virus were infected into a neuroblastoma cell line, IMR-32 and a human glioblastoma cell line, GBM 8401, respectively. To determine the permissiveness of these virus strains in both cells, the severity of the cytopathic effect (CPE), relative quantitative analysis with hemadsorption and hemagglutination, reverse transcriptase-polymerase chain reaction (RT-PCR) as well as kinetic study of viral protein synthesis were performed.

Results: All tested viruses grew well in IMR-32, but only B/3143/Taiwan/97 replicated well in GBM 8401, according to the results of CPE, hemagglutination, hemadsorption, RT-PCR and viral protein synthesis.

Conclusions: Besides the binding of viral receptor and hemagglutinin being critical for a permissive infection, the interaction of other virus proteins and the other unknown host factors might also affect the ability of influenza B virus to infect a host cell.

Key words: Influenza B virus, tumor cell line, viral growth, virulence

Introduction

Influenza viruses are important human pathogens that cause mainly upper and lower respiratory infection. Influenza A and B viruses both cause epidemics in Taiwan every year. In addition to respiratory syndromes, central nervous system (CNS) involvement of the viruses has also been reported. For instance, Reye's syndrome has been well documented as a serious complication of influenza B virus infection [1]. Other neurological symptoms such as acute encephalitis and encephalopathy are the most common clinical presentation of CNS caused by both influenza A and B viruses [2,3]. Although neurovirulent variants, i.e., A/WS/33(H1N1), A/PR/8/34(H1N1), A/Japan/57 (H2N2), and B/Lee/40 could be produced by continued passages in the laboratory, the mechanism by which the influenza virus invades the CNS has still not been proved by isolation of infectious virus particles from CNS [4]. One hypothesis is that neurotropism of the particular strains is due to changing of certain antigenic determinants.

In the period 1997-1998, several cases of influenza A and B involving CNS diseases were reported in Japan [2,3]. At that time, we reported that two strains of influenza B isolated in 1997 also co-circulated in Taiwan. One of them was recovered from a meningoencephalitic patient [5]. At the same time, a case of acute influenza B virus encephalitis was also reported [6]. Subsequently, we also recovered an influenza B virus from cerebrospinal fluid (CSF) of a six-year old boy who had been suffering from non-cleaved cell lymphoma and had been receiving chemotherapy for almost one

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year. In this study, we used a neuroblastoma cell line (IMR-32) and a human glioblastoma cell line (GBM 8401) to discriminate the infectivity of the influenza B virus isolated from patients who have central nervous involvement or respiratory symptoms.

Methods

Virus strains

The influenza B virus designated as B/Taiwan/2027/99 (2027/99) was isolated from the CSF of a six-year old boy. This patient had been diagnosed as having mild psychomotor retardation with speech delay when he was 3.5 years old. At 5 years of age (August 1997), he suffered from progressive right tonsillar enlargement and received a biopsy. The pathology revealed small non-cleaved cell lymphoma. Chemotherapy regimens were administered, including vincristine, prednisolone, epirubicin, cyclophosphamide, methotrexate, mercaptopurine, as well as intrathecal methotrexate, hydrocortisone, and cytosine arabinoside. The chemotherapy was completed on July 30, 1998.

On January 26, 1999, he suffered from cough, rhinorrhea, and generalized weakness. Consciousness disturbance with mouth angle deviation to the left was also noted on January 27, 1999. Neurological examination was normal except for a mild bilateral decrease of muscle power in the lower limbs. CSF was sanguinous with around 150 erythrocytes per high power field. Glucose concentration in CSF was normal (81 mg/dL) but there was slightly elevated protein concentration (92 mg/dL). Brain computed tomography scan was essentially normal except for mild brain atrophy. B/Taiwan/3143/97 (3143/97), and B/Taiwan/21706/97 (21706/97) were the previously published strains [5]; and B/Taiwan/2026/99 (2026/99) and B/Taiwan/2195/99 (2195/99) were isolated from throat swabs of the patients who were diagnosed with pneumonia [7]. All virus strains were propagated in Madin-Darby canine kidney (MDCK) [Bioresource Collection and Research Center (BCRC) 60004] cells and stored at –80°C.

Cell culture

The MDCK cells were cultured with Eagle's medium with 10% fetal calf serum. The glioblastoma cell line GBM 8401 (BCRC 60163) and the neuroblastoma cell line IMR-32 (BCRC 60014), were purchased from the BCRC, Taiwan, and were cultured with 90% Roswell Park Memorial Institute medium 1640 and 10% fetal calf serum.

Hemagglutination test

For testing neurotropism, 100 50% tissue culture infectious doses ($TCID_{50}$) per mL of each virus were infected into the IMR-32 or GBM 8401. At 48 h after infection, the culture fluid was separated from the cell monolayers, followed by hemagglutination (HA) test, which was done at room temperature with 0.5% guinea pig erythrocytes, as described previously [8]. Both cell-associated and cell-free viral hemagglutinin genes were also detected by reverse transcriptase-polymerase chain reaction (RT-PCR).

Hemadsorption assay

The hemadsorption (HAd) assay was performed at 48 h after infection, as described previously [9]. Briefly, infected cells were incubated with 0.5% guinea pig erythrocytes which had been prepared with phosphate-buffered saline (PBS, pH 7.4). Following incubation at 4°C for 20 min, the cells were washed with PBS three times to remove unbound erythrocytes. Five fields were chosen randomly and the numbers of erythrocyte rosettes formed were counted using light microscopy.

RNA extraction

Virion RNA was extracted using the commercialized RNA isolation reagent, TRI Reagent™ LS (Molecular Research Center Inc., Cincinnati, OH, USA) as described previously [10]. Briefly, one volume (0.25 mL) of virus-infected cells was mixed with three volumes (0.75 mL) of TRI Reagent™ LS. After the sample was homogenized, 0.2 mL of chloroform was added and the sample was shaken vigorously for 15 seconds and then kept at room temperature for 2 to 3 min. The resulting mixture was centrifuged at 12,000 *g* for 15 min. The aqueous phase was collected; the viral RNA was precipitated using isopropanol and then washed with 70% ethanol. Finally, the purified RNA was dissolved with 9 μL sterilized de-ionized water.

RT and PCR

The cDNA synthesis and PCR amplifications of the coding regions of the hemagglutinin-1 domains in the hemagglutinin genes were carried out using primers corresponding to nucleotides 403-423 (5'-AATCTTCTC AGAGGATATGAA-3') and 961-937 (5'-GGCAATCTG CTTCACCAATTAAAGG-3'), yielding a 559-bp fragment, according to a previously described procedure [5]. Briefly, 9 μL of the RNA preparation was mixed with 16 μL of a buffer containing 50 mM potassium chloride (KCl), 10 mM Tris-hydrochloride (Tris-HCl)

[pH 8.4], 2.5 mM magnesium chloride ($MgCl_2$) and 0.02% gelatin, 1 mM each of deoxyribonucleotide triphosphate, 2 units of ribonuclease (RNase) inhibitor, 50 pmol of forward primer and 1 μL of avian myeloblastosis virus reverse transcriptase (AMV RT) [RNase H minus; Promega, Madison, WI, USA] 5-10 unit/μL. The mixture was incubated at 42°C for 60 min to make cDNA. For PCR, 100 μL of reaction mixture containing amplification buffer (10 mM Tris-HCl) [pH 8.3], 50 mM KCl, 2.5 mM $MgCl_2$, 0.02% gelatin), 20 pmol each of the primers, 1 μL (4 units) of KlenTaq DNA polymerase (Clontech, Palo Alto, CA, USA) and 25 μL of cDNA solution. The amplification reaction was performed at 94°C for 0.5 min, 55°C for 30 sec, and 72°C for 1 min for 30 cycles in a DNA thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer, Foster City, CA, USA). One-tenth of the amplified product was applied to 1.5% agarose gel and stained with ethidium bromide. The PCR products on agarose gel electrophoresis revealed a band of 562 base pairs visible under an ultraviolet illuminator.

Kinetic study of viral protein synthesis in glioblastoma cell line

GBM 8401 cells were infected with influenza viruses at 100 $TCID_{50}$ per mL and adsorbed for 2 h at 37°C. Unadsorbed virus particles were washed with PBS three times. After virus adsorption, cell culture medium was replaced by the methionine-depleted medium at 1, 3, 5, 7, 9 and 16 h, and then starved for 30 min. The cells were labeled with [^{35}S]methionine/[^{35}S]cysteine (100 μCi per mL) for 1 h. Unlabeled isotope was washed with cold PBS. The cell suspension was collected into a 1.5-mL microcentrifuge tube and was centrifuged at 3000 rpm for 5 min. The cell pellet was resuspended in 50 μL 2X sodium dodecyl sulfate (SDS) gel-loading buffer (100 mM Tris HCl [pH 6.8]; 200 mM dithiothreitol; 4% SDS; 0.2% bromophenol blue and 20% glycerol). Each sample was boiled for 10 min and proteins were analyzed by SDS-polyacrylamide gel electrophoresis with a 12.5% acrylamide concentration [11].

Results

To test the infectivity of the influenza B virus in different cell lines, 5 tested viruses were infected into the neuroblastoma cell line IMR-32 and the glioblastoma cells GBM 8401, respectively. All the viruses could produce an irregular shaped cytopathic effect (CPE) in IMR-32 cells (Fig.1). In contrast, only 3143/97 produced a CPE in GBM 8401 (Fig. 2A). No CPE was observed in other tested viruses at 48 h after infection (Fig. 2B). In order to examine whether the infected cells produced virus particles, HA test was performed on the culture fluid. The HA result indicated that only cells infected with 3143/97 show a HA titer of >160 (Table 1). For other virus strains, the HA titer was undetectable. Similar results were shown in other virus strains, including 21706/97, 2026/99, and 2195/99 (Table 1). With regard to CPE in GBM 8401, only 3143/97 showed diffused irregular cell shape through the monolayer of the infected cells and also a positive HA test result (Fig. 2C and Table 1). This result suggested that 3143/97, but not 2027/99, could affect the glioblastoma cells directly.

In order to further confirm the infectivity, all the tested viruses were subjected to HAd assay with guinea pig erythrocytes after the viruses infected GBM 8401 cells. The results indicated that the guinea pig erythrocytes could form varying numbers of rosettes on the surface of virus-infected cells with all the viruses (Fig. 3A-F). Coinciding with the results of CPE, 3143/97 had the highest number of rosettes, with 71.4 per field (Table 1). The second highest was 21706/97, with an average value of 28.8 rosettes per field. Comparing the 1999 isolates separately, 2027/99 had the highest number of rosettes (13.8 on average) followed by 2026/99 (9.2 on average) and 2195/99 (7.4 on average) [Table 1].

To test whether the progeny viruses can be formed and released from the GBM 8401 cell, the virus-infected cell and its culture medium were separated and the hemagglutinin genes were detected by RT-PCR. The results indicated that hemagglutinin gene was abundant in the culture medium of the cells infected with 3143/97 (Table 1). Trace amounts of the gene were also detected in the media when the cells were infected with 21706/97 and 2027/99 individually (Table 1). Negative results occurred in viruses 2026/99 and 2195/99. In contrast, hemagglutinin genes were abundant inside infected glioblastoma cells, with all the tested viruses (Table 1). These findings indicated that, although all the tested viruses could replicate in the glioblastoma cell line, progeny viruses could only be released from 3143/97 at high concentration. In contrast, there were only traces or undetectable amounts of cell-free virus in this cell line.

Only 3143/97 could synthesize the main virus proteins in glioblastoma cells (hemagglutinin, nucleoprotein, non-structural protein 1 and matrix

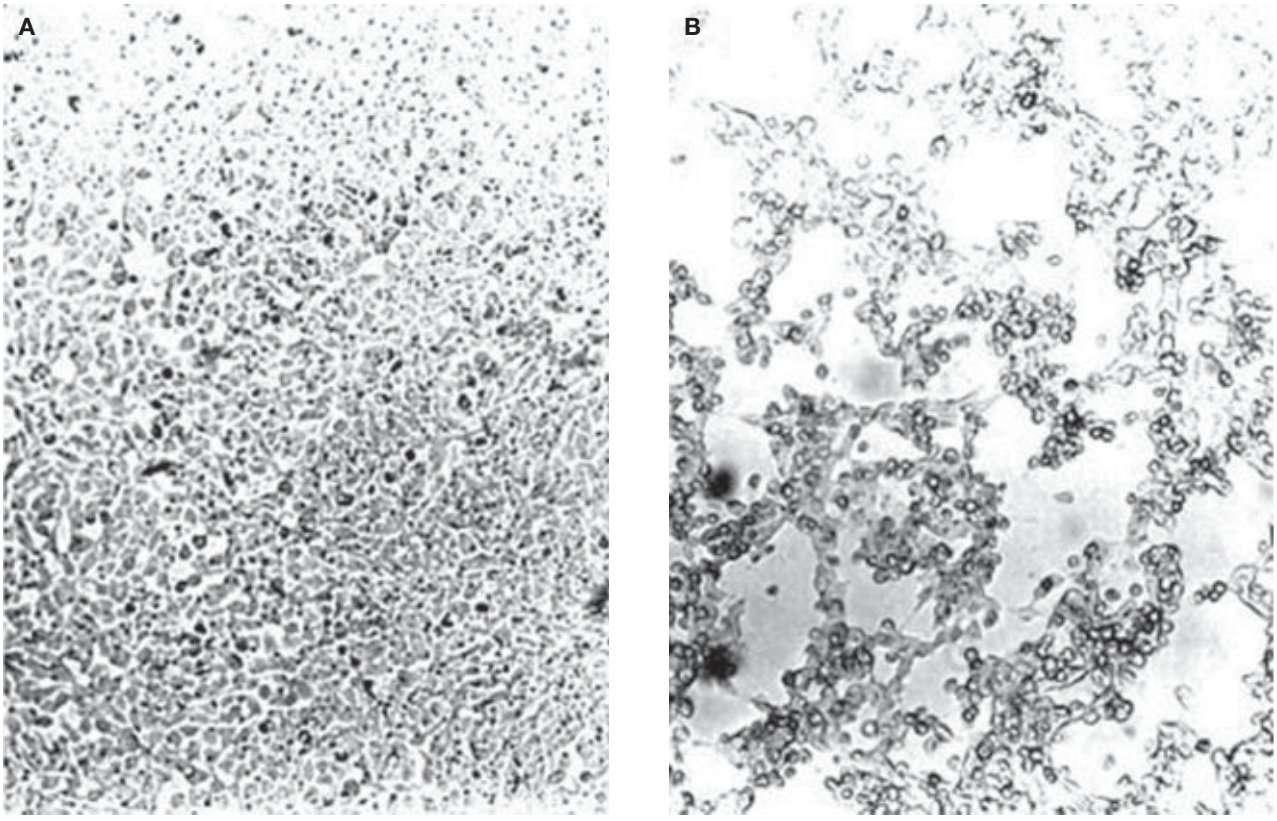

Fig. 1. Cytopathic effect (CPE) of the human neuroblastoma cell line IMR-32 infected with influenza B virus. A) Confluent monolayer of uninfected IMR-32 cell line (× 100). B) IMR-32 shows irregular shape of CPE when infected with influenza B virus.

protein) at the early stage of infection (Fig. 4A). The newly synthesized protein could be detected at 5 h postinfection, with maximum levels at 9 h postinfection (Fig. 4B).

Discussion

Influenza-associated encephalopathy or encephalitis is not rare in children and has been recently reported in Japan

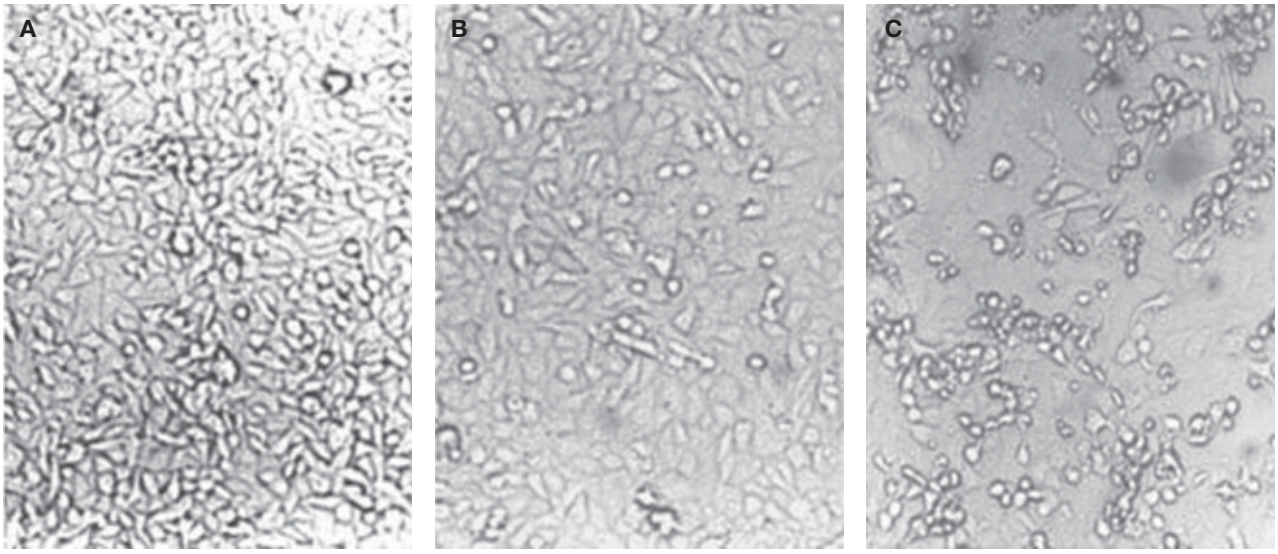

Fig. 2. Cytopathic effect of the human glioblastoma cell line, GBM 8401 infected with influenza B virus. A) Confluent monolayer of uninfected GBM 8401 cell line (× 100). B) GBM 8401 infected with either B/Taiwan/21706/97, B/Taiwan/2027/99, B/Taiwan/2026/99 or B/Taiwan/2195/99. C) GBM 8401 infected with B/Taiwan/3143/97.

Table 1. Evaluation of the neurotropism of influenza B isolates in GBM 8401 cells

Virus strain	CPE	HA test	Hemadsorption assay (average no. of rosettes per 5 field) [× 200]	RT-PCR of hemagglutinin gene	
				Supernatant	Cell
3143/97	+	>160	71.4[a]	++[b]	++++
21706/97	–	<20	28.8	±	++++
2026/99	–	<20	9.2	–	++++
2027/99	–	<20	13.8	±	++++
2195/99	–	<20	7.4	–	++++

Abbreviations: CPE = cytopathic effect; HA = hemagglutination; RT-PCR = reverse transcriptase-polymerase chain reaction; + = present; – = absent; ± = low intensity; ++ = medium intensity; ++++ = high intensity

[a]Average number of rosettes counted per field.

[b]Undetectable.

[2,3,6,12-14]. Review of case reports from the 1998/1999 influenza season in Japan showed that at least 217 cases of encephalopathy or encephalitis were associated with influenza-like illness, over 80% of which were in children under 5 years of age [15]. In almost all reported cases of encephalitis caused by influenza viruses, the RNA rather than the virion was detected from CSF in influenza A/B viruses. In practice, it has been difficult to culture the virus from the CSF although it has been reported for three Jamaican patients, two of whom had encephalitic signs [16].

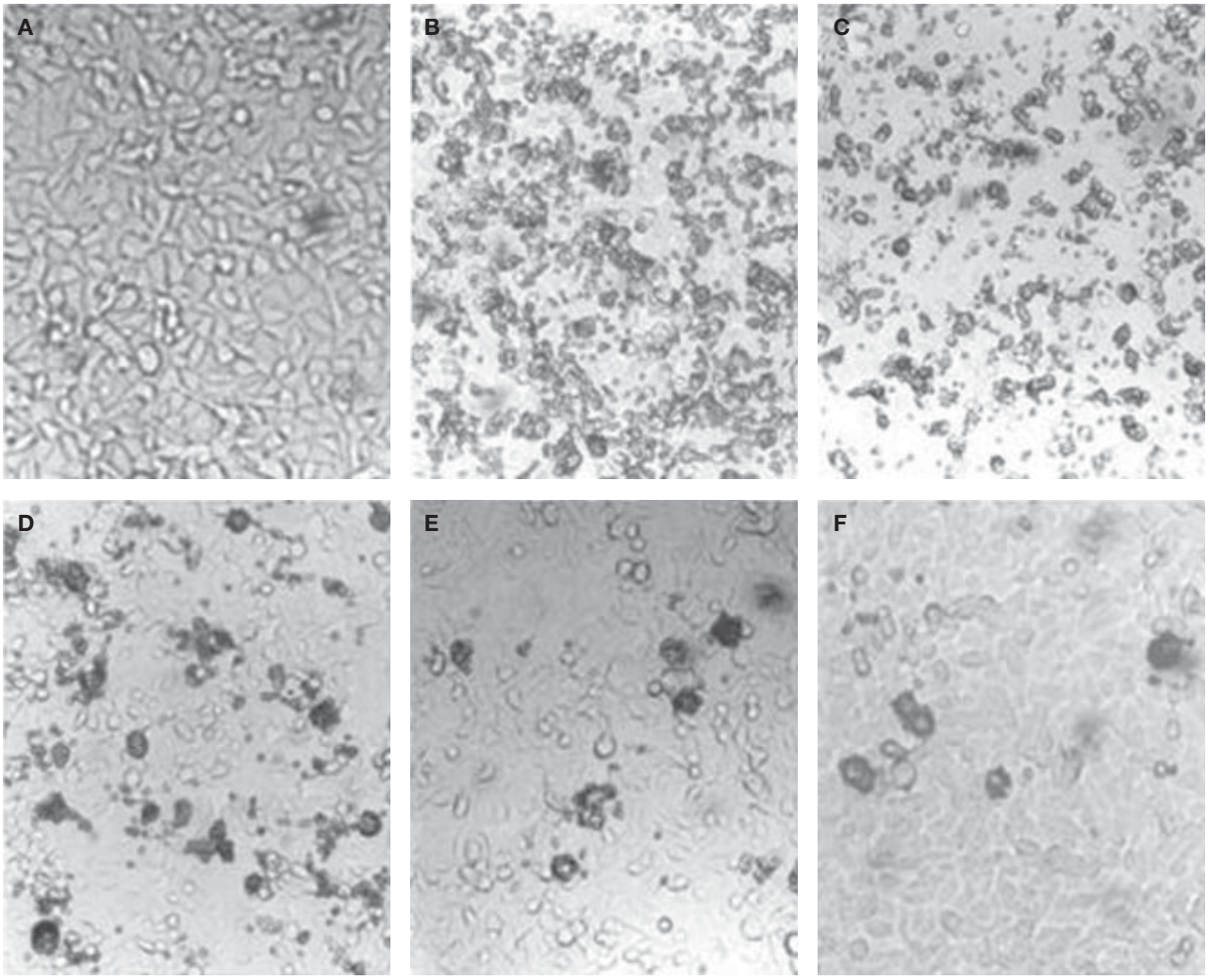

Fig. 3. Hemadsorption test of GBM 8401 infected with influenza B isolates (× 100). A) Control (mock infection). B-F) B/Taiwan/3143/97, B/Taiwan/21706/97, B/Taiwan/2027/99, B/Taiwan/2026/99 and B/Taiwan/2195/99, respectively.

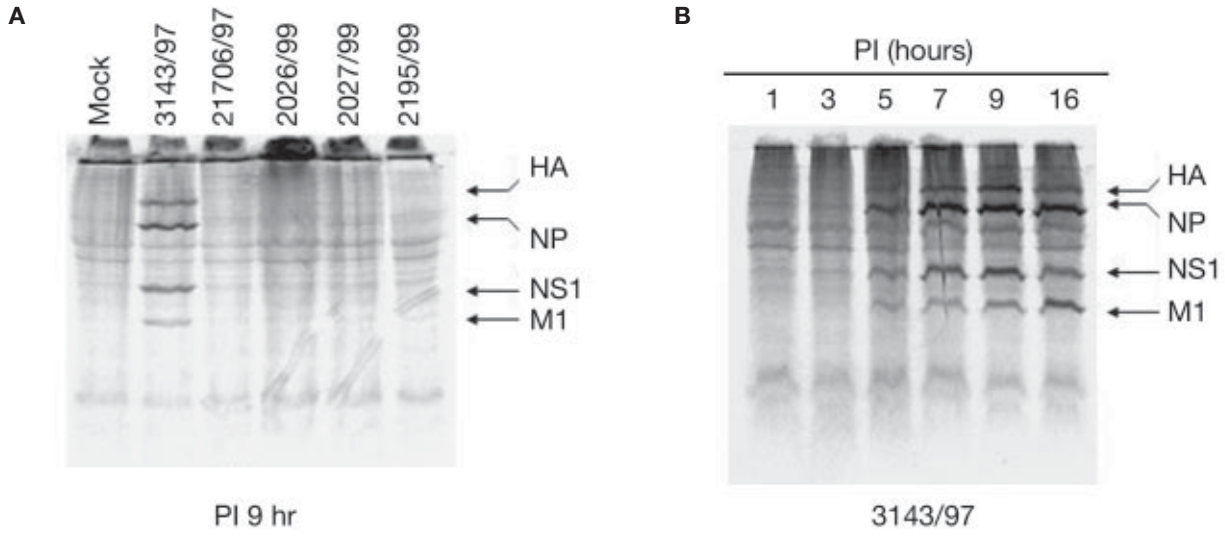

Fig. 4. Kinetic study of protein synthesis of influenza B virus in GBM 8401 cells. A) Viral protein synthesis in GBM 8401 after infection for 9 h. B) Time course of GBM 8401 infected with B/Taiwan/3143/97.

One of our influenza B viruses, 2027/99, was directly isolated from the CSF sample. This result suggests that the influenza B virus might shift its tissue tropism from affecting the respiratory system to both the respiratory and nervous systems. This might be because 2027/99 was recovered from the patient who was diagnosed as having small non-cleaved cell lymphoma and treated with chemotherapeutic drugs for almost one year. Although the chemotherapy had been stopped six months previously in that patient, the host's immunity might not have recovered to normal. For this reason, once infected with the influenza virus, it might be possible for the virus to overcome the host's immune system and invade the CNS.

To assay the infectivity of different strains of influenza B viruses, we chose two cell model systems, a neuroblastoma cell line, IMR-32, and a glioblastoma cell line, GBM 8401. IMR-32 was established from a neuroblastoma occurring in a 13-month-old Caucasian male [17]. This cell line has been used frequently as a tool for antiviral research and viral receptor studies with various species of viruses that often caused neurological symptoms. IMR-32 was demonstrated to be susceptible to various neurotropic viruses like coxsackie B3, poliovirus, varicella-zoster virus, Newcastle disease virus, measles virus, JC virus, rabies virus and herpes simplex virus [18-24]. Our results showed that IMR-32 was also susceptible to the influenza B viruses despite their degree of neurotropism. This suggests that influenza B virus has the potential to infect the CNS, but the pathway by which the virus enters the CNS is still unknown. Although we could not use IMR-32 to differentiate the neurotropism, this cell line might be able to act as an alternative tool for the study of virus and nervous cell interaction in future.

GBM 8401 was established from a Chinese female with brain glioblastoma multiforme [25]. This cell line had been sub-cultured for more than 100 passages during 24 months in vitro [25]. In contrast to IMR-32, there has been no previous report of use of GBM 8401 to study any virus. Our study is the first model to use GBM 8401 to evaluate the infectivity of influenza B viruses. The positive results of HAd showed that all the tested viruses could express HA. However, when we used cell-free supernatants to assay HA titer, only 3143/97 produced a high titer on the HA test. This result indicates that 3143/97 could infect GBM 8401 cells permissively. In contrast, other tested strains of influenza B viruses could infect GBM 8401 cells only in an abortive manner, although both 21706/97 and 2027/99 could also produce detectable amounts of viral particles. The kinetic study of viral protein synthesis in GBM 8401 cells showed that only 3143/97 could synthesize viral proteins in the cells in the early period of infection.

In conclusion, our results indicate that the glioblastoma cell line GBM 8401 together with HAd provide a simple method to differentiate the infectivity of influenza B virus. Previous alteration of the host's immune system by administration of a combination of steroids might provide an opportunity for the virus to invade the CNS despite neurotropism. Further animal study is required to establish how the influenza B virus enters the CNS.

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