Isolation of Nanjing Local Strains of HHV-7 and Their Biological and Immunological Characteristics

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To investigate the biological and immunological characteristics of the Nanjing local strains of HHV7, four strains of herpesvirus were isolated from saliva specimens of one healthy individual and three children suffering from a kidney disease in Nanjing. The viruses were identified by transmission electron microscopy (EM), indirect immunofluorescence assay (IFA) with a specific monoclonal antibody; nested polymerase chain reaction, restriction mapping and DNA sequencing. The virus-infected cells showed the typical cytophathic effect (CPE) under microscopy and could be detected by IFA with the human herpesvirus-7 (HHV-7) specific antibody. Under EM, herpesvirus-like and virions capsids could be found in their cytoplasm or nucleoplasm. HHV-7 DNA fragments amplified from infected cells by nested PCR were confirmed by restriction mapping and DNA sequencing. Similarly to DC strain, an known HHV-7 strain used in the present study as the positive control, the virus could be inactivated by ultraviolet irradiation for 10 min, heated at 45°C for 30 min, pH < 5 or > 9 at 4°C for 2 h and ether or chloroform for 10 h. The virus induced the production of TNF-α, IL-10 and IL-12p70 while inhibited IFN-γ secretion, increased the percentage of $CD2^+$ cells while decreased that of $CD4^+$ or $CD45RA^+$ cells. The results indicate that the viruses isolated in Nanjing are HHV-7, which has similar biological characteristic to the known HHV-7 strain, DC. Infection with HHV-7 *in vitro* could affect immune function of lymphocytes by disturbing cytokine production and CD antigen expression. *Cellular & Molecular Immunology*. 2004;1(5):367-372.

Key Words: HHV-7, DNA sequence, cytokine, CD

Introduction

Human herpesvirus 7 (HHV-7), a new type of lymphotropic human herpesvirus, was firstly discovered in 1990 (1), after HHV-6 was isolated in 1986 (2). HHV-7 showed a high detection rate in the transplanted recipients (3) and the patients with chronic fatigue syndrome (4) or immunodeficiency (5). So far no other diseases were proved to be caused by HHV-7 infection except of exanthem subitum (6, 7). HHV-6 and HHV-7 can all be reactivated at any time if host immune system becomes defective. From the phenomena that the reactivated HHV-6 can cause a variety of clinical problems, such as exanthems along with interstitial pneumonia or hepatitis，have an influence on the course of autoimmune and proliferate diseases such as systemic lupus erythematosus and Hodgkin's disease, HHV-7 was also deduced to be associated with similar disorders when it was reactivated (8).

On the other hand, HHV-7 was found to bind CD4 molecule of T lymphocytes competitively with human immunodeficiency virus (HIV) (9). Some researchers suggested that HHV-7 could be used as a new vector for the gene therapy of AIDS (10). Therefore, intensive research should be carried out on HHV-7 for little was known about it. For example, once HHV-7 infects $CD4^+$ T lymphocyte (1, 3, 9), what is the effect of HHV-7 infection on body immune function? Is it safe when used as a vector *in vivo*? Although HHV-7 can be isolated in many area of the world, no reports on HHV-7 isolation in China has appeared. In this study we isolated the local strain of HHV-7 in China firstly and described its biological and immunological characteristics in detail.

Materials and Methods

Virus

DC strain, a HHV-7 strain from Glasgow University, United Kingdom (15), was a gift kindly given by Dr. Peiris (Department of Microbiology, the University of Hong Kong, China). DC strain was used in this study as a positive control and propagated in SUPT1 cell-line cultures.

Cells

Cord blood mononuclear cells (CBMCs) were obtained from heparin-anticoagulated and Hank's sol-diluted cord

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blood of health newborn babies by centrifuging with Ficoll-Hypague lymphocyte separating medium. CBMCs (2×10^9 cells/L) were cultured at 37°C, 5% CO_2 in completed medium (CM), RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin (all from GIBCO, USA), containing 40 mg/L PHA (Sigma, USA) and 10^4 U/L IL-2 (Nanjing Institute of Military Medical Science, Nanjing, China).

SUPT1 cells, a T-lymphocyte-line susceptible to HHV-7 (11) provided by Dr. Peiris of the University of Hong Kong, was grown in the CM mentioned above. L929 cells, used as the target cells in cytotoxicity test for TNF-α, were also cultured in the CM.

Agents

Monoclonal antibody KR-4 specific to HHV-7 (7, 12) was kindly provided by Professor Yamanishi Koichi (Medical School, Osaka University, Japan). Monoclonal antibodies to CD2, CD3, CD4, CD8, CD11a and CD45RA molecule and APAAP kit were products of Beijing BangDing Biol. Co. (Beijing, China). Monoclonal neutralization antibody to TNF-α was provided by Professor Boquan Jin (the Fourth Milit. Med. Univ., Xi'an, China). FITC-labeled sheep anti-mouse IgG was a product of Shanghai Huamei Biol. Co. (Shanghai, China). Detecting kits of IFN-γ, IL-10 and IL-12p70 were purchased from Genzyme Co. (USA). PCR kit (FDDK-2) was the product of Shanghai Fuhua Biol. Co. (Shanghai, China). The kit for DNA purification was from Bio-RAD Co. (USA) and restriction enzyme EcoR I was from MBI Co. (Lithuania).

PCR primers

The nested-PCR primers were synthesized in the Cell Institution, Chinese Academy of Medical Science (Shanghai, China). The sequences of the primers were P1: 5'-TAT CCC AGC TGT TTT CAT ATA GTA AC-3'; P2: 5'-GCC TTG CGG TAG CAC TAG ATT TTT TG; P3: 5'-CAT CCA GAA ATG ATA GAC AG-3'; P4: 5'-AGG AGA ATT CTG TAC CCA TG-3' (3, 13).

Virus isolation and identification

The saliva samples (1 ml each) from two healthy individual and five children suffering from kidney diseases were incubated with the cultured CBMCs preactivated by PHA and IL-2. The CBMCs were showing balloon-like cytopathic effects (CPE) (1, 12) were collected for smearing and performed indirect immunofluorescence assay (IFA) for HHV-7 specific antigen by using monoclonal antibody KR-4 and FITC-labeled sheep anti-mouse IgG. The CPE showing cells were also detected for virions in them with transmission electron microscopy (EM) by the routine method (3, 12).

Virus DNA analysis

Lysate of infected SUPT1 cells (5 ml) obtained by repeated freeze-thaw cycles were incubated with the cytolytic medium containing proteinase K at 55°C for 1 h, and then at 95°C for 10 min to inactivate proteinase K. DNA was extracted by chloroform-phenol method and dissolved in pH8 TE and then stored at -20°C for further use. A 186 bp sequence of HHV-7 was amplified by the first step PCR in a DNA Thermal Cycler (PE2400) with using nested PCR according to the literature (3, 13). PCR products were separated by eletrophoresis on an 8% polyacrylamide gel and then visualized under ultraviolet. The product of first PCR steps was purified according to the kit manual. The purified PCR product was digested by EcoR I at 37°C for 4 h followed by an electrophoresis on 2% agarose gel. The DNA sequence analysis of the first PCR product was performed by Shanghai Genecore Co. (Shanghai, China).

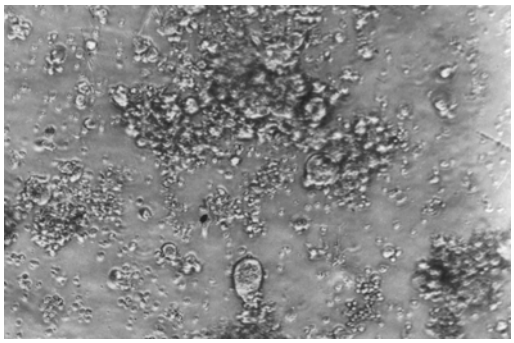

Figure 1. After cultured for 10 days, the CBMCs inoculated with the saliva sampler containing HHV-7 present the ballooning and polykaryotic CPE (200 ×).

Biological characteristic analysis

The culture supernatant of SUPT1 inoculated with virus was treated with different conditions as following: ultraviolet irradiation for 10, 20 and 30 min, heating at 45°C, 56°C and 60°C for 30 min, storing in pH3, pH5, pH9 and pH10 at 4°C for 120 min and incubation with ether or chloroform at 4°C for 12 h. The infection activity was analyzed by observing the typical CPE of the SUPT1 cells inoculated with the treated supernatant.

Immunological characteristic analysis

CBMCs inoculated with the virus and were cultured for six days. By centrifuging, infected CBMCs were separated from the supernatants. TNF-α in the supernatants was measured by the microcytotoxicity test (14) with mono-layer of L929 cells and neutralization antibody to TNF-α. IFN-γ, IL-10, and IL-12p70 were determined by sandwich ELISA according to the kit direction. The re-suspended CBMCs were smeared and then tested for CD antigen expression by the method of APAAP staining, following the kit direction.

Statistical analysis

Statistical determinations were made by Mann-Whitney U test. Two-sided *p* values are presented in all experiments and significance were defined as $p < 0.05$.

Results

Virus isolation and identification

Four strains viruses were isolated from saliva specimens of one healthy individual and three children suffering from kidney diseases. All of these four strains could propagate in CBMCs and SUPT1 cells and show the typical balloon-like CPE (Figure 1). The average time of CPE appearance was

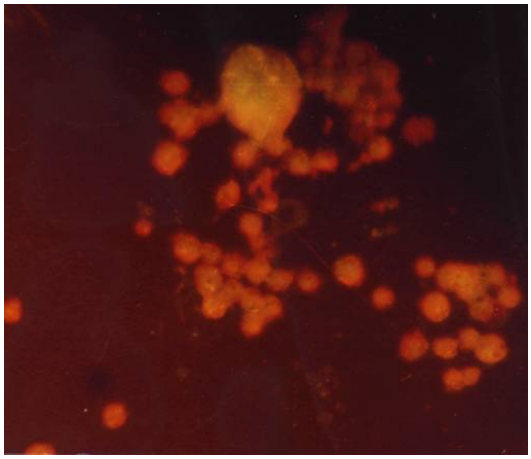

Figure 2. HHV-7 infected cells show bright greenish-yellow fluorescence after co-incubated with KR-4 McAb to HHV-7 and stained with FITC-labeled sheep anti-mouse IgG.

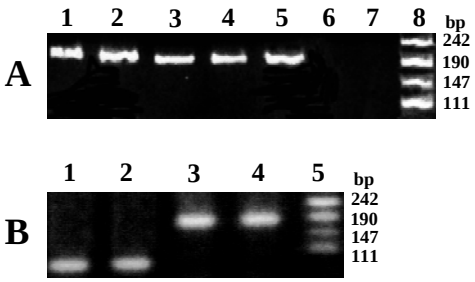

Figure 4. HHV-7 or DC strain DNA detection in inoculated CBMCs. (A) PCR detection of HHV-7 DNA in inoculated CBMCs. Lane 1, YY1; Lane 2, YY2; lane 3, YY5; lane 4, YY6; lane 5, DC strain; lane 6, uninoculated CBMCs; lane 7, HSB2 infected with HHV-6 GS; lane 8, DNA marker. (B) Nested-PCR detection of YY5 and DC strain DNA in inoculated CBMCs. Lane 1, nested PCR products of DC strain; lane 2, nested PCR products of YY5; lane 3, first PCR products of DC strain; lane 4, first PCR products of YY5; lane 5, DNA marker.

8-10 days and the percentage of CPE-showing cells at its peak were about 15%. Following the subculture, the average time of CPE appearance shortened to 3-5 days and the percentage of CPE-showing cells reached 30%. These four strains couldn't infect HSB2 cell which is susceptible to HHV-6 (15) and human embryo lung diploid cell line, which is susceptible to HCMV. In IFA, the infected cells showed bright greenish-yellow fluorescence under fluorescence microscope (Figure 2), just as the standard DC strain-infected cells did. These four strains were named as YY1, YY2, YY5, and YY6. With transmission electron microscopy, herpesvirus-like virions could be found in cytoplasm of the infected SUPT1 cells or infected CBMCs (Figure 3). At the same time, nucleocapsids and capsids without any visible core could be found in the nucleoplasm and cytoplasm as well. The virions showed an envelope 180 nm in diameter and the capsids were about 100 nm in diameter (Figure 3).

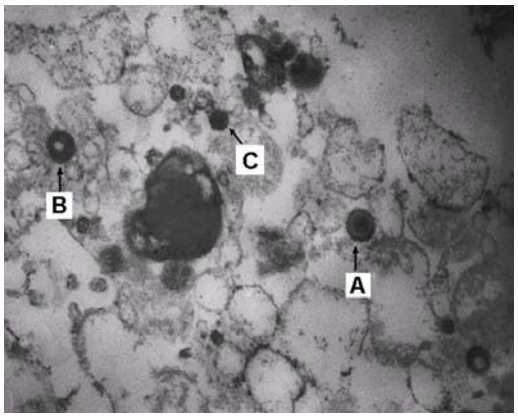

Figure 3. Herpesvirus-like particles in the cytoplasm of infected CBMCs under electron microscopy (30,000 ×). Arrow A shows mature virus particles containing an electron-dense core. Arrow B shows the virus without any visible core. Arrow C shows a nucleocapsid.

Virus DNA analysis

The expected product amplified from DNA samples of both the YY5 strain infected cells and the DC strain infected cells showed a bright band at 186 bp after the first step PCR and at 92 bp after the second step PCR (Figure 4). The 186 bp product could be digested by EcoR I into two fragments: one was 126 bp, the other was 60 bp (Figure 5). DNA equencing showed an identification of 100% between the sequences of the 186 bp fragment and the homologous gene of HHV-7 (RK strain 19852~20037, Gene Bank No. AF037218 and JI strain 15631~15816, Gene Bank No. U43400). This sequence is as follows: 5'-TAT CCC AGC TGT TTT CAT ATA GTA ACA TTA CCA ATT CAG TTT TCA TCC AGA AAT GAT AGA CAG ATG TTG GTG TCA AGC TAT CCT AAT GAA GGC TAC TTT GAA GTA CAA ATG TGC CCA TGG GTA CAG AAT TCT CCT CTT CAA ATT GTT ATT AAA TCT TTT TCA AAA AAT CTA GTG CTA CCG CAA GGC-3'.

Biological characteristics

The viruses treated with ultraviolet irradiation, heating at 45°C, 56°C and 60°C, storing in pH 3, pH 5, pH 9 and pH 10 and incubating with ether or chloroform were incapable of inducing CPE on SUPT1 cells.

Immunological characteristics

TNF-α activities of YY5 strain-infected CBMCs supernatants and DC strain-infected CBMCs supernatants were significantly higher than that of control CBMCs

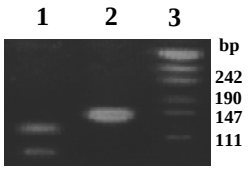

Figure 5. EcoR I digest the 186 bp product in two fragment 126 bp and 60 bp. Lane 1, two fragment after EcoRI digest; lane 2, first PCR products of YY5; lane 3, DNA marker.

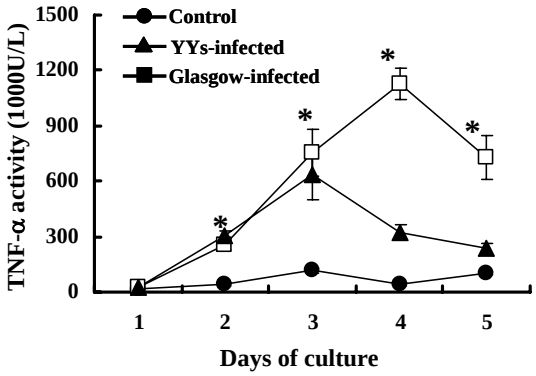

Figure 6. Kinetics of TNF-α in CBMCs culture supernatants (n = 12). * $p < 0.05$, compared with the control.

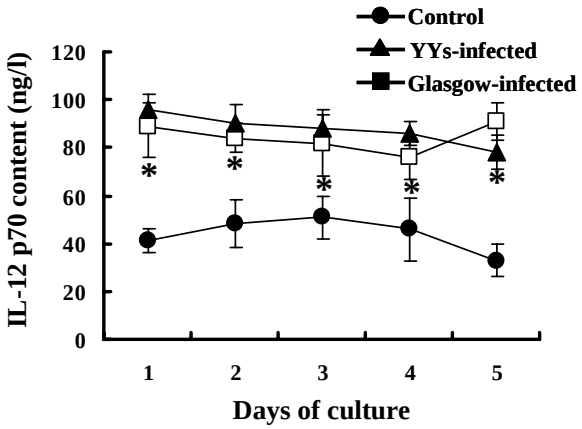

Figure 8. Kinetics of IL-12p70 production of CBMCs induced by HHV-7 (n = 12). * $p < 0.05$, compared with the control.

supernatant at different day after infection. HHV-7-infected CBMCs culture supernatants reached a maximal level of TNF-α activity at 3 (DC strain) or 4 (YY5 strain) days after infection. The kinetics curves of TNF-α were shown in Figure 6. Neutralization test showed monoclonal antibody to TNF-α could neutralize all of the cytotoxic activity of the supernatants, indicating that the cytotoxic is really induced by TNF-α.

IL-10 (ng/L) contents in the supernatants of CBMCs infected by YY5 or DC strain were remarkably higher than in the supernatants of control CBMCs at 3, 4 and 5 days after infection and there was no difference between the IL-10 contents in the supernatants of CBMCs infected by YY5 or DC strain. The dynamic characteristic of IL-10 induction by HHV-7 was presented in Figure 7. IL-12p70 contents (ng/L) of the CBMCs supernatants infected with YY5 or DC strain were also higher than those of the control CBMCs supernatants at different day after infection. The IL-12 level induced by YY5 strain was similar to that induced by DC strain. The dynamic curves of IL-12 were shown in Figure 8.

IFN-γ contents (ng/L) of both the CBMCs supernatants infected with YY5 and with DC strains were slightly lower than those of control CBMCs supernatants, but no statistical difference existed. The kinetics of IFN-γ production was shown in Figure 9.

Compared with the control CBMCs, the percentage of CD2 antigen positive cells increased, while that of CD4 positive cells or CD45RA positive cells decreased in both infected CBMCs with YY5 and with DC strain of HHV-7. Moreover, the ratio of CD4 to CD8 reduced from 2.1 ± 0.4 of CBMCs control to 1.5 ± 0.3 of YY5-infected CBMCs or 1.3 ± 0.4 of DC-infected CBMCs. No significant changes in statistics were found in the percentages of CD3, CD8 and CD11b positive cells in CBMCs infected with HHV-7. The data were all displayed in Table 1.

Discussion

HHV-7 strains were firstly isolated from human peripheral blood mononuclear cells (PBMCs) (1, 3). Afterwards, HHV-7 isolations from human saliva specimens were reported. Ihira M reported HHV-7 shedding in saliva was isolated in 92 (34.1%) of the 270 saliva samples obtained (22). Freitas RB reported HHV-7-specific IgM and/or IgG antibodies were found in 190 (51.4%) of the patients suffering from exanthematous illnesses in Belem, North Brazil by indirect immunofluorescence assay (IFA) while the HHV-7 DNA could not be detected in sera examined by PCR/nested PCR (23). So far HHV-7 is considered to exist

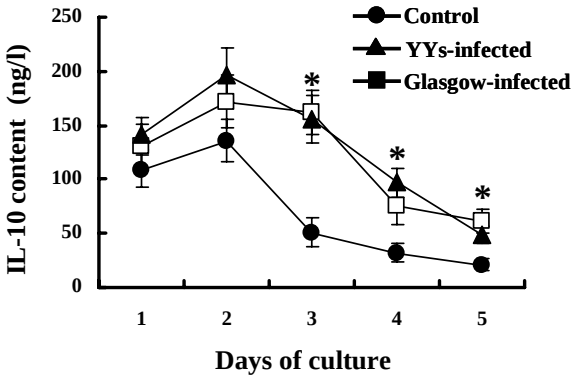

Figure 7. Kinetics of IL-10 production of CBMCs induced by HHV-7 (n = 12). *$p < 0.05$, compared with the control.

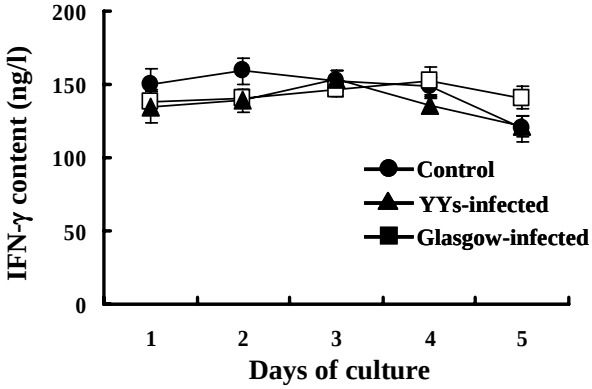

Figure 9. Kinetics of IFN-γ production of CBMCs induced by HHV-7 (n = 12).

Table 1. *Effects of infection with HHV-7 on CD antigen expressions of CBMCs (n = 6).*

Group	CD2	CD3	CD4	CD8	CD11b	CD45RA
Control	61.2 ± 5.09	73.4 ± 3.79	46.3 ± 2.34	22.5 ± 4.19	16.8 ± 3.27	72.9 ± 4.4
YY5 infection	72.6 ± 5.01*	73.6 ± 3.43	32.3 ± 1.97	22.1 ± 4.20	15.8 ± 3.27	27.6 ± 10.58*
DC strain infection	72.5 ± 3.45*	78.3 ± 3.46	29.0 ± 2.10	23.6 ± 6.29	18.0 ± 3.21	26.1 ± 3.85*

Percentage (%) of CD antigen positive cells cultured for 48 h, *$p < 0.05$, compared with the control.

generally in human saliva and to be transmitted mainly by saliva (5, 12, 17, 18). That is why we chose saliva as sources for HHV-7 isolation.

The isolated viruses are proved to be strains of HHV-7. The evidences are as follows: 1. The virus infection could induce typical balloon-like CPE on CBMCs and SUPT1 cell-line, but did not on HSB2 cell line, which is susceptible to HHV-6. 2. With EM, herpesvirus-like virions, 180 nm in diameter, were found in infected cytoplasms and capsids, 100 nm in diameter, in the nucleoplasms, which is identical with the morphological characteristic of HHV-7 reported previously (3, 12). 3. The cells infected with the virus showed a clear and definite positive reaction to monoclonal antibody specific to HHV-7. 4. The expected 186bp products of the first step and 92 bp of the second step were gained by the nested PCR based on the primers specific to HHV-7, EcoR I digested the 186 bp product into two fragment of 126 bp and 60 bp (3, 19) and the sequence of the 186 bp product coincide with the HHV-7 sequence we have known.

The biological characteristic analysis proved that the viruses isolated were susceptible to inactivation effects of ether or chloroform. This characteristic coincides with the fact that HHV-7 has an envelope. It is easy to inactivate the virus by ultraviolet irradiation, heating at 45^{o}C, or pH > 9.

Although it has been reported that HHV-7 could induce cytokines production in leukocytes (20), it is not well known how HHV-7 affects cytokine network balance. In this study, HHV-7 was proved to promote TNF-α, IL-10 and IL-12 productions in CBMCs cultures. The enhancing effects of HHV-7 on TNF-α and IL-12 production of CBMCs were very obvious and persistent. However, the much stronger TNF-α secretion enhancing activity of YY5 strain than that of DC strain demonstrated that the local strains was indicating the differences between different strains. As for IL-10, the effect of HHV-7 infection was to delay the reduction IL-10 secretion. While the slightly inhibitory effect of HHV-7 infection on IFN-γ production was quite uncertain. On the other hand, the results of this study that HHV-7 infection inhibited the expressions of CD4 and CD45RA while slightly increased CD2 expression in CBMCs. Only the inhibitory effect of HHV-7 infection on CD4 expression had been reported heretofore (9, 21). Now it might be conjectured that infection by HHV-7 could perturb immune function by disturbing cytokine production and expression of CD antigen.

Acknowledgements

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