

Full Length Research Paper

A novel polyclonal antibody against human cytomegalovirus: General characteristics and potential application in diagnosis

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There is no vaccine available for human cytomegalovirus (HCMV) infection and the treatment is very limited; therefore, it is still an important cause of morbidity and occasional mortality in transplant recipients. An HCMV peptide was selected based on the GenBank sequence M60929. The peptide was conjugated with keyhole limpet haemocyanin and used to immunise New Zealand rabbits to prepare polyclonal antibody. After immunisation, the serum of the rabbits was obtained and purified. ELISA, immunoprecipitation and immunocytochemical staining were used to identify antibodies and antibody titre. The identification of the synthetic peptide antibody was confirmed by ELISA, immunoprecipitation and immunofluorescence against HCMV Towne and AD169 strains. The antibody can be used to detect the HCMV infection by immunocytochemical staining. The synthetic peptide showed favourable immunogenicity. The serum from immunised rabbits could be used to identify HCMV AD169 and Towne strains. Future research should be directed to epitope screening of synthetic HCMV peptides, which could help to understand HCMV infection and virus-neutralising antibodies more fully and to prepare HCMV vaccines and antiviral drugs.

Key words: Human cytomegalovirus, AD169 strain, Towne strains, polyclonal antibody.

INTRODUCTION

Although, different assays have been developed and introduced to diagnostic virology laboratories for detection and monitoring of human cytomegalovirus (HCMV) infection and several significant advances have been made in its prevention and treatment, HCMV is still an important cause of morbidity and occasional mortality in immunocompromised patients.

Glycoprotein B (gB) is the major envelope glycoprotein of HCMV and it is encoded by UL55 gene which belongs to the open reading frames (ORF) of HCMV genome. HCMV gB has been implicated in host cell entry, cell-to-cell virus transmission and fusion of infected cells, as well as being an important target for humoral and cellular immune responses (Coaquette et al., 2004; Zhou et al.,

2007). gB has also been shown to be a major target for the production of neutralising antibodies and antibodies to gB comprise 40 to 70% of the total neutralising activity against HCMV (Yu et al., 2003; Britt and Mach, 1996). HCMV subtype classification has been based most frequently on gB (Puchhammer-Stöckl and Görzer, 2006). HCMV gB is expressed as a precursor molecule that is glycosylated and then cleaved at codon 461 to form a disulphide-linked complex of gp55 and gp116 (Coaquette et al., 2004). Peptide variation in gp116 is strongly clustered at precise regions of the protein (Zhou et al., 2007). Based on the N terminus of gB (gBn), HCMV can be classified into four major subtypes (Coaquette et al., 2004; Zhou et al., 2007). Many studies have reported a correlation between the gB genotype and the occurrence of CMV associated disease in immunocompromised patients (Tarragó et al., 2003; Torok-Storb et al., 1997; Humar et al., 2003; Fries et al., 1994). Different gB subtypes can exhibit different cell

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tropism and different levels of neurotropism (Puchhammer-Stöckl and Görzer, 2006; Tarragó et al., 2003; Meyer-König et al., 1998a, b). In a previous study, 145 (77.5%) plasma samples were gBn1-positive in 187 gB-positive patients (Fan et al., 2009). Other studies in Asia found that, gBn1 was the most frequent genotype (Kashiwagi et al., 2002; Yu et al., 2006).

Recently, antibodies to CMV gB were studied by several scholars (Rasmussen and Cowan, 2003; Zhang and Pass, 2004; Yu et al., 2003). However, there was no breakthrough. In this study, a peptide from gBn1 was synthesised, which was used to immunise New Zealand rabbits, from which antiserum was prepared. It was demonstrated that the synthetic peptide had favourable immunogenicity. In the future, more research should be dedicated to screening epitopes in the synthetic peptide, which could help in understanding HCMV and its neutralising antibodies and to prepare HCMV vaccine and antiviral drugs.

MATERIALS AND METHODS

Cell lines and reagents

HCMV AD169, Towne strain and human embryonic lung fibroblasts (MRC-5) were purchased from the American type culture collection (Manassas, USA). Healthy male New Zealand rabbits were provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Complete and incomplete Freund's adjuvants were both from Sigma (USA). Mouse monoclonal antibody against CMV and the horseradish peroxidase (HRP)-labelled sheep anti-rabbit IgG were the product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). The protein G-Sepharose and ECL reagent were purchased from Pierce and Cell Signaling Technology (USA), respectively.

Synthesis of peptide and preparation of immunogens

According to the published GenBank sequence M60929, the amino acid sequence (AGTS ATHS HHSSH TTSA HSRSG SVSQR VTSSQ TVSHG V) of gBn1 antigen peptide was determined and synthesised by Zhongtai Biotechnology Company (Hangzhou, China). They provided and confirmed the following parameters: the molecular weight of the synthetic peptide was 3998.2 Dalton and the purity as analysed by reverse-phase HPLC was 95.1%. To enhance the immunogenicity of the peptide, some of the peptides were cross-linked with keyhole limpet haemocyanin (KLH). The synthetic peptide was preserved at -20°C.

Production and purification of antibody

The synthetic peptide was used as an immunogen to immunise New Zealand rabbits. In the initial immunisation, the synthetic peptide with KLH was mixed with an equal volume of Freund's complete adjuvant. In the four booster immunisations (2-week intervals), immunogens were the synthetic peptide with KLH that were mixed with an equal volume of Freund's incomplete adjuvant. After the third booster immunisation, a small amount of blood from the rabbits' ear veins was taken. By ELISA, with the synthetic peptide as antigen, the antibody titres in the serum of immune rabbits reached more than 1:1000, which showed a perfect immune response. Three days after the fourth booster immunisation, carotid

artery blood was removed from the immunised rabbits. The serum was separated from the blood and preserved at -80°C with aseptic packaging. The affinity purification method (protein G-Sepharose) was used to purify the rabbit serum.

Preparation of HCMV protein samples

MRC-5 cells were infected with HCMV Towne or AD169 strain at an MOI of 10:1. Cells were cultured for 3 days. The infected cells were collected and lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM sodium EDTA, 1 mM EGTA, 1% Triton, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM leupeptin, 1 mM PMSF) for 30 min at 4°C. The insoluble material was then removed by centrifugation at 8000 \times g for 10 min at 4°C. The concentration of protein in each cell lysate was determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard.

ELISA assay

Each well of 96-well high-binding EIA plates (Corning, USA) was coated with 10 ng of HCMV Towne strain, AD169 strain and MRC-5 cells overnight at 4°C in PBS. After two washes with PBS and blocking with 5% skimmed milk and PBS for 1 h at room temperature, the plates were incubated with anti-serum (1:500 to 1:50,000 dilution in 5% skim-milk PBS) for another hour at room temperature. Then, anti-mouse IgG-HRP was added and the wells were developed with 3,3',5,5'-tetramethylbenzidine (TMB). The plates were read in an ELISA plate reader at 450 nm.

Immunoprecipitation and western blotting

Immunoprecipitation of HCMV or other proteins from cellular lysates was performed using mAb followed by western blotting. Cellular proteins (200 μ g) were immunoprecipitated overnight with the prepared rabbit anti-HCMV antibody (2 μ g per sample) coupled with protein G-Sepharose beads. After washing, samples were boiled at 100°C for 4 min and then, separated in 10% SDS-PAGE under reducing conditions. The proteins were transferred into the PVDF membrane (0.45 μ m; Millipore, Bedford, MA, USA) and blocked with 1% BSA in TBS-T buffer. Western blotting was carried out using rabbit anti-HCMV antibody or other specific antibodies, followed by HRP-conjugated secondary antibodies. The reaction was developed with enhanced ECL reagents and analysed by the VersaDoc5000 imaging system (Bio-Rad).

Immunofluorescence

MRC-5 cells were cultured on slides (Lab-Tek Chamber Slide System; Nalge Nunc International) and infected with HCMV Towne or AD169 strain for 72 h at an MOI of 10:1. The cell was fixed with 4% paraformaldehyde at 4°C for 20 min and incubated with anti-HCMV mAb at room temperature for 1 h. The cells were further incubated with FITC-coupled secondary antibody (Santa Cruz Biotechnology) for 30 min. Fluorescence was detected by FACScan flow cytometry (Becton Dickinson, San Jose, CA, USA) and photographed by fluorescence microscopy (Olympus IX81; Tokyo, Japan).

Immunocytochemical staining

In a previous studies, HCMV gBn genotyping using real-time quantitative PCR was established successfully (Fan et al., 2009).

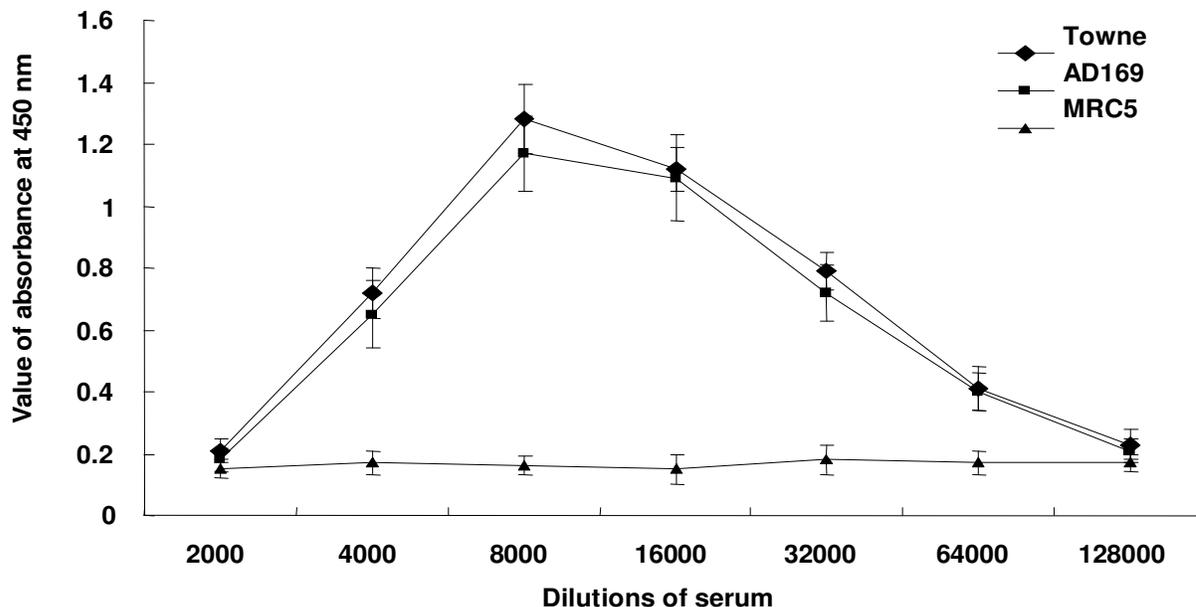


Figure 1. The protein samples of HCMV Towne strain, AD169 strain and MRC-5 analyzed by ELISA. The protein samples of HCMV Towne strain, AD169 strain and MRC-5 as antigens were coated in a 96-well plate and the purified serum was used as the primary antibody (1:1000, 1:16000, 1:64000, 1:256000, 1:1024000, respectively). HRP- labeled sheep anti-rabbit IgG mAb (1:5000) was used as the second antibody. The results were measured at 450 nm with ELISA reader (Bio-Rad).

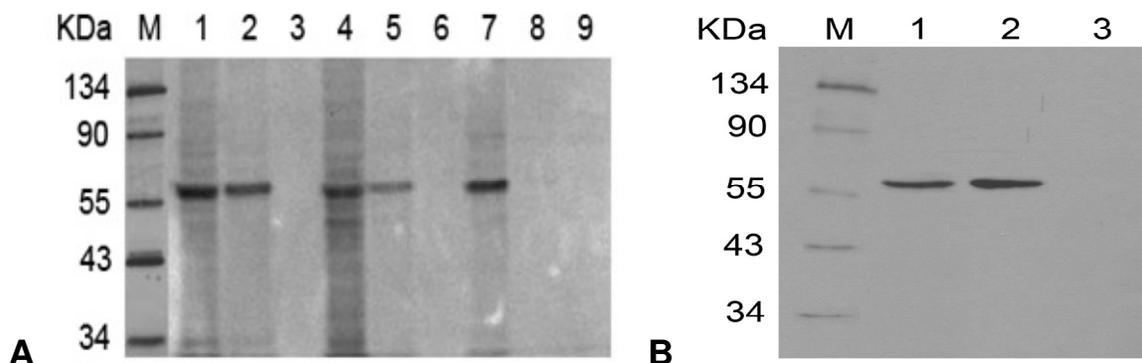


Figure 2. Confirmation of the specificity of the antibody to HCMV by western-blot. (A), Interaction with HCMV Towne strain, AD169 strain and MRC-5 cells. MRC-5 cells and its infecting HCMV Towne strain, AD169 strain were lysed in lysis buffer. 100 ug cell protein lysates per sample were mixed with 2 × SDS loading buffer containing DTT and heated at 100°C for 10 min before resolving by SDS-PAGE. Proteins were transferred to a PVDF membrane. Western-blot was performed using rabbit anti-HCMV antibody as primary antibody, visualized by the ECL reaction and analyzed by VersaDoc imagine system (Bio-Rad); (B), controls were provided in order to confirm the specificity of the antibody to HCMV using a mouse monoclonal antibody against CMV (Santa Cruz) as primary antibody. Lane 1, HCMV Towne strain (40 µg); lane 2, HCMV AD169 strain (40 µg); lane 3, MRC-5 (40 µg); lane 4, Towne strain (20 µg); lane 5, AD169 strain (20 µg); lane 6, MRC-5 (20 µg); lane 7, Towne strain (10 µg); lane 8, AD169 strain (10 µg); Lane 9, MRC-5 (10 µg).

Peripheral blood mononuclear cells from persons who had been confirmed as HCMV-gBn-positive by real-time PCR were fixed on slides. HCMV-negative samples were determined by immunocytochemical staining and PCR. The purified serum was used as the detecting antibody followed by reagents from the DAKO Envision System (Dako, Denmark). The immunocytochemical staining was performed with routine procedures as described by the

manufacturer's protocol. Cells that were stained yellow or brown were deemed positive, whereas those stained blue were deemed negative.

Data analysis

All the experiments were performed in triplicate.

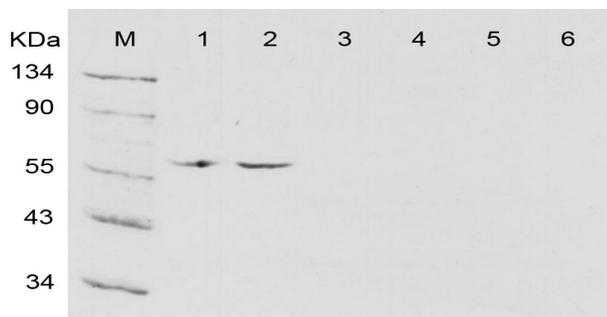


Figure 3. Confirmation of the specificity of the antibody to HCMV by Immunoprecipitation. Samples from cellular lysates were subjected to Immunoprecipitation using rabbit anti-HCMV antibody (lanes 1, 2 and 3) or normal rabbit IgG (lanes 4, 5 and 6). Western-blot was performed using a mouse monoclonal antibody against CMV (Santa Cruz) as primary antibody and visualized by the ECL reaction. Lane 1 and 4, HCMV Towne strain; lane 2 and 5, HCMV AD169 strain; lane 3 and 6, MRC-5.

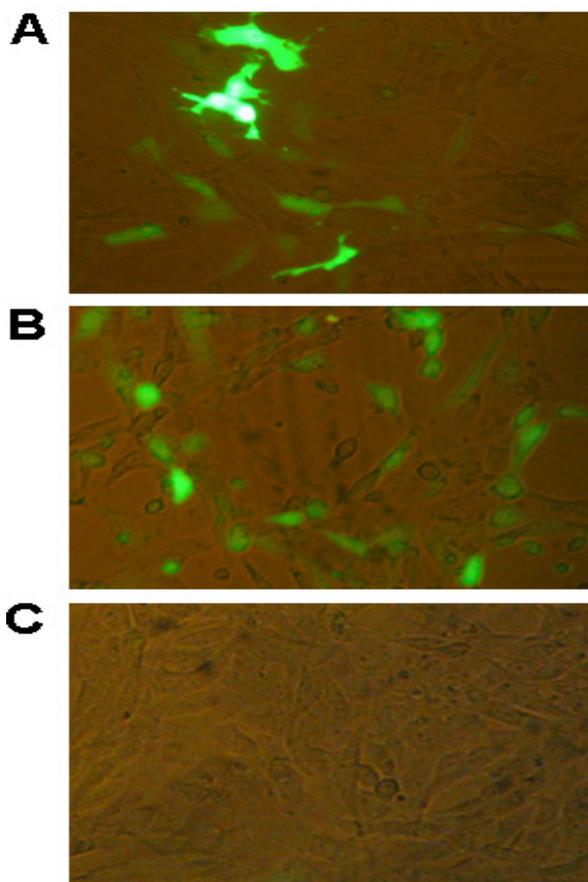


Figure 4. Interaction of the serum with HCMV Towne strain and AD169 strain in Immunofluorescent analysis. HCMV AD169 (A), Towne (B) and MRC-5 cells (C) were cytospinned and fixed by cold acetone, respectively. Rabbit anti-HCMV antibody was added to each sample followed by FITC labeled-sheep anti-rabbit IgG. Then, these were photographed and observed under the fluorescence microscope (magnification $\times 200$).

RESULTS

Identification of the synthetic peptide antibody against HCMV

The identification of the synthetic peptide antibody was confirmed by ELISA, western-blot, immunoprecipitation and immunofluorescent analysis against HCMV Towne and AD169 strains. (Figures 1 to 4)

Initial clinical application of the synthetic peptide antibody

Previously, blood samples were identified by PCR as gB-positive or gB-negative. In the meantime, the samples were determined by immunocytochemical staining. Several positive cells were found in the gBn-positive samples, whereas there were no positive cells in the gB-negative samples (Figure 5).

DISCUSSION

HCMV remains an important cause of morbidity in immunocompromised persons, especially in transplant recipients. HCMV gB is the major envelope glycoprotein of the virus. It is a major target of neutralising antibodies and is considered to be a major candidate for an HCMV vaccine (Puchhammer-Stöckl and Görzer, 2006). In the past years, there were many researches on CMV-specific antibodies (Iizuka et al, 2003; Plotkin, 1999; Rasmussen and Cowan, 2003; Yu et al., 2003; Zhang and Pass, 2004). Although, the antibodies corresponding to the HCMV epitopes had several advantages, there are also various disadvantages which led to failure of CMV vaccine.

There are a number of reasons for thinking that gB responses might provide immunity and play an important role in such immunity (Plotkin, 1999). Recently, there were many studies on gB and these have mainly focused on the association of gB subtypes and clinical outcomes. The association of gB subtypes and immune response, exposure history, type of infection, geographic distribution, HCMV disease and prognosis are still not well understood. Also, in the process of research on HCMV vaccine and immunogenicity of antigens, the titer of protective antibodies were not very effective, which was due to insufficient studies on polymorphism of epitopes. Therefore, the screening of epitopes in gB was considered as of great significance and further studies on gB could help the understanding of the pathogenetic mechanism of HCMV.

Previous studies have shown that, gBn1 is the most popular genotype in gB. Therefore, in this study, gBn1 peptide was synthesized based on GenBank sequence M60929 and was used to immunise New Zealand rabbits. The titre of the antiserum from immunised rabbits was

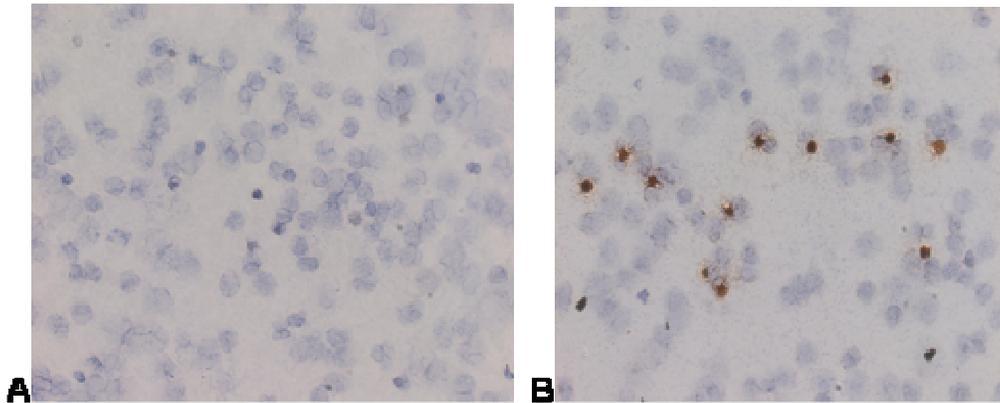


Figure 5. Immunohistochemical application of the serum in peripheral blood mononuclear cells. Peripheral blood mononuclear cells infecting gB and negative control were fixed on slides, rabbit anti-HCMV antibody was added to each sample followed by reagents from the DAKO envision system kit (DAKO). The reaction was developed by substrate DBA. Cell staining was photographed by BH-2 Olympus microscope (magnification $\times 40$). (A), Negative control; (B), sample of gBn positive.

1:64000. With ELISA, immunoprecipitation and immunofluorescence, the serum was shown to interact with HCMV Towne and AD169 strains. However, the serum may possess the potential application in HCMV diagnosis.

When the peptides from gBn1, gBn2, gBn3 and gBn4 were coated in a 96-well plate, the result for gBn1 peptide was obviously different from the other peptides; therefore, it was considered that the antiserum could distinguish the linear structure of gB peptide from its three-dimensional structure. Simultaneously, by immunocytochemical staining, the plasma with HCMV was verified by the antiserum.

In summary, the synthesized gBn1 peptide was shown to be interacted with HCMV Towne and AD169 strains. In the future, more research should be directed to epitope screening in the synthetic peptides, which could help in understanding the neutralising antibodies to HCMV and prepare HCMV vaccines and antiviral drugs.

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