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Original Article

Detection of Herpes Simplex Virus Antibodies Using the Whole Virus and Recombinant gD

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Abstract

Background and Aims: Herpes simplex virus type 1 (HSV1) remains a potentially serious health problem world wide. All infected people, including asymptomatic ones, are potential sources for virus transmission. Virus envelope contains at least 13 glycoproteins, which glycoprotein D is the major target of immune responses. The aim of this study was development of a specific method that is a more rapid, sensitive and specific test compared to the virus neutralization test which is applied as gold standard test.

Methods: In this study, the Western blot technique using crude HSV1 whole particle and baculovirus derived glycoprotein D of HSV1 as antigens was set. Human sera were analyzed by virus neutralization test and then serum samples with reciprocal virus neutralization antibody titers of 32, 64, and 128 were taken to be analyzed by Western blotting.

Results: It was shown that there was a very good correlation between results obtained from virus neutralization antibody titers and those of Western blotting. Western blotting using recombinant glycoprotein D of HSV1 as an antigen showed positive results similar to the whole HSV1 antigen.

Conclusion: Our study showed the Western blotting using recombinant glycoprotein D can replace virus neutralization test in diagnosis of HSV1 infections.

Keywords: Glycoprotein D; Herpes simplex virus type1; Recombinant gD; Virus neutralization test

Introduction

Herpes simplex virus type 1 (HSV1) is an enveloped, double-stranded DNA virus and is one of the most important pathogen of human. It causes a broad range of infections from asymptomatic to life threatening encephalitis. After primary infection, the virus becomes latent in the trigeminal ganglia and persists for the life time (1-4).

The virus genome encodes more than 30 proteins, of which 13 known antigenically distinct glycoproteins are located in the envelope of the virions (5-7). These glycoproteins are the primary inducers of humoral immune response in HSV infection and are also important targets of cell-mediated immune responses (8, 9). Glycoprotein D (gD) is involved in virus infection and pathogenesis (10-14). It is also a suitable candidate for investigation because gD is a confirmed target of neutralizing antibodies, antibody dependent

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cell mediated cytotoxicity (ADCC), and virus-specific cellular responses mediated by both CD4+ and CD8+ T lymphocytes (15).
In this study, we tried to run Western blotting using HSV1 whole particles and baculo virus derived HSV-gD1 as antigens and compared the results with virus neutralization test (VNT).

Methods

Cell culture

HeLa cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% heat inactivated FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37ºC with an atmosphere of 5% CO2.

Virus propagation

Herpes simplex virus (HSV) was isolated from labial vesicles of a patient and identified as HSV1 using monoclonal antibody (15). HeLa cells were infected at a multiplicity of infection (moi) of 1 pfu/cell, after one hour adsorption, unabsorbed virus suspension was discarded and the cells were washed two times by sterile phosphate buffer saline (pH=7). Serum free DMEM containing 100 IU/ml penicillin and 100 µg/ml streptomycin was added to the cells. After appearance of cytopathic effects (CPE) in almost 70% of the cells, virus was harvested by freezing and thawing. Cell debris was removed by centrifugation at 8000g for 30 min at 4ºC and the supernatant was used as whole antigen in Western blotting.

Virus neutralization test (VNT)

One hundred serum samples were collected from volunteers with or without history of herpetic disease and used for detection of neutralizing antibodies. Briefly, two fold dilutions of heat inactivated of each serum ranging from 1/2 to 1/256 were prepared in serum-free DMEM. Each dilution was mixed with 100TCID50 of HSV1 and kept for 1h at 37°C in a total volume of 200 µl. Duplicate 100 µl samples were added to HeLa cells in 96-well micro titer plates. DMEM containing 2% heat inactivated newborn-calf serum was added to all inoculated cells including positive and negative controls and were incubated at 37°C. The neutralization titer of each serum was considered as: the reciprocal titer of the highest dilution preventing CPE in inoculated cells.

Preparation of recombinant glycoprotein D of HSV1-gD

Plaque purified recombinant baculo virus containing full-length gD-1 gene with titer of 10^7- 10^8 pfu/ml was applied to infect Spodoptera frugiperda clone 9 (Sf9) insect cells. The cells were grown in Grace's medium containing 10% fetal bovine serum. In order to get a suitable culture of the cells, supplementation of the growth medium with 3.5 g/l yeast extract and 3.3 g/l Lactalbumin Hydrolysate was necessary. Gentamycin sulfates (50 mg/ml) and penicillin (100 IU/ml) were also added, and the pH was adjusted to 6.2.
Sf9 cells were inoculated with purified recombinant baculovirus carrying full length of gD-1 gene at multiplicity of infection of 1 and incubated at 27ºC for 5 days. The supernatant of the infected cells was collected, filtered through a 0.45 µm pore size filter and stored at -80 ºC. In order to produce recombinant gD-1, Sf9 cells were inoculated with purified virus at multiplicity of infection of 5 and incubated at 27 ºC for 96h. The cells were harvested and washed three times with ice-cold sterile phosphate buffer (pH 7). The cells were subjected to freezing and thawing for 3 times then were sonicated at 20KHZ, 5 times for 30 seconds(1, 16). The lysate was first treated with CHAPS (0.1mM) and PMSF and then electrophoresed under denaturing conditions and in order to confirm gD-1, Western blotting was done using monoclonal anti HSV1-gD (8).

Purification of HSV1 by sucrose density gradient centrifugation

Vero cells infected at a multiplicity of infection (moi) of 1 pfu/cell. After appearing the CPEs, the virus was harvested and purified on sucrose density gradients. Briefly, cell debris were removed by centrifugation at 8000g and 4°C for 30 min and the virus was concentrated by centrifugation at 100000 g for 2 hr. The pellet was re-suspended in TNE buffer, pH 7.5 (0.1 M Tris-HCl, 0.1 M NaCl, 0.001 M EDTA) and then purified on step gradient consisting of 30%, 40%, 50% and 60% of sucrose in TNE

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and centrifuged at 75000 g for 5 hr. Purified virus was collected, dialyzed and stored at -80°C (16).

Immunoblotting

Virus seeds (either purified virus by sucrose density gradient centrifugation and recombinant gD-1 protein were mixed separately with electrophoresis sample buffer (50mM Tris pH 8.6, 10% glycerol, 2% W/V sodium dodecyl sulfate, and 0.1 % bromophenol blue) and boiled for 10 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (PAGE) was performed in 12% polyacrylamide and the separated proteins were transferred to nitrocellulose paper. The proteins bonds on nitrocellulose were reacted with total human HSV1 antibody after blocking in 5% gelatin. Two fold dilutions of positive and negative serum samples for neutralization antibody were tasted by Western blotting. The bonds were detected by reacting the blots with alkaline phosphate- conjugated anti human antibody and visualized by diaminobenzidine (DAB) as substrate (15).

Results

HSV1 specific neutralizing antibodies

Micro neutralization test was performed using 100 TCID50 of the wild-type HSV1. Virus neutralization titer was the reciprocal of the highest dilution of the serum that completely inhibited CPE in the inoculated cells. Data in Table 1 shows that no HSV1 specific neutralizing antibody (titer < 8) was detected in 11% of the tested sera, while 89% had neutralizing antibody against HSV1.

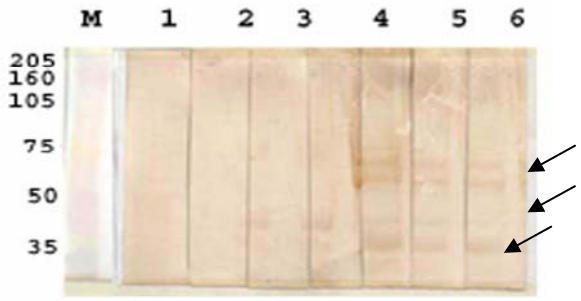


Fig. 1: Western blotting results for gD1 purified protein. Its bands are indicated with arrows.

SDS-PAGE and Western blot analysis

The SDS-PAGE result of the whole virus is showed in Figure 1. The virus specific bands cannot be differentiated from the cellular proteins by SDS-PAGE method. Since some cellular protein have similar molecular weight to viral proteins, therefore Western blotting was used to differentiate these proteins.

Western blot analysis using whole virus as an antigen

Table 1. Virus neutralization antibodies to the HSV1 in tested sera.

Virus neutralization antibody titer*	≤2	4	8	16	32	64	128	≥256
Percentage of positive sera **	7%	4%	2%	0%	15%	24%	22%	26%

* Virus neutralization titer was expressed as the reciprocal of the highest dilution that completely inhibited virus CPE in inoculated cells

** Two fold dilutions of 100 serum samples were tested against 100 TCID50 of the HSV1.

Table 2. The number of positive and negative sera in indicated dilution by VNT and Western blot.

A

Western blotting results \ VNT* Results	Positive	Negative	Total
Positive	7	0	7
Negative	1	7	8
Total	8	7	15

* VNT, virus neutralization test

B

Western blotting results \ VNT* Results	Positive	Negative	Total
Positive	15	1	16
Negative	1	5	6
Total	16	6	22

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All of the serum samples with neutralization antibody titers of 32, 64, and 128 were used in Western blotting. Starting with 1000, two fold dilutions were made from the tested sera by VNT and subjected to Western blotting, using whole virus and recombinant HSV-gD1 protein. Since PAGE results showed a non specific band, it was confirmed by Western blotting.

Western blot analysis using recombinant glycoprotein D of HSV1 (gD-1)

Western blot analysis of baculovirus infected cells showed four distinct bands with apparent molecular weight of ~51,~43.5,~33, and 31 KDa that reacted with monoclonal anti gD-1 antibody (fig.1). The serum samples with positive results in Western blotting using HSV1 were positive at dilutions of 1000, 2000, and 4000 using recombinant gD-1 as antigen (fig. 1).

Comparison of VNT and Western blotting

In this study, in order to control confounding effects (such as: age, sex …), the same serum samples were used in both VNT and Western blotting. We performed MC Nemar's test to compare results of VNT and Western blotting (Table 2).

The dilutions of 1000 (A), 4000 (B) of tested sera in WB and 32(A), 128(B) in VN antibody titers were chosen to show their statistical differences, respectively.

Comparing the results as shown in table 2, there is no statistically significant difference.

Discussion

Herpes simplex virus type 1 (HSV1) is a human pathogen which causes a variety of diseases including cold sores, eye and genital infections,

neonatal infections and encephalitis (3). Different methods are used for HSV antibody detection including ELISA, IF, CF and virus neutralization test (VNT). Virus neutralization test is more specific than other serological tests (17). This method requires cell culture and skilled personal and takes long time to perform.

We tried to optimize Western blotting test using HSV1 whole particles and baculovirus derived HSV1-gD.

Based on the results of MC Nemar's statistical test, differences between the two tests are not statistically significant ($p>0.05$). Western blotting has many advantages compared with VNT and can detect low titers antibody in

In this study, 100 serum samples with reciprocal virus neutralization antibody titers of 32, 64, and 128 were chosen and dilutions of 1000, 2000, and 4000 of the sera were prepared and tested by Western blotting, using whole HSV-1 and HSV-gD recombinant proteins. Similarly positive and negative sera were used as controls.

patient's serum. Considering these advantages, Western blotting can be used instead of VNT with similar sensitivity and specifity.

Using recombinant baculo derived HSV1-gD as antigen in Western blotting showed similar results compared with using whole virus. We believe that recombinant proteins should be used instead of whole infectious virus in Western blotting and other serologic tests.

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