Lab.med. **16**: 327–331 (1992)

Detection of Herpes simplex Virus (HSV) type specific Antibodies by a microtechnique Western blot assay

Nachweis von Herpes simplex Virus (HSV) typenspezifischen Antikörpern mit einem Mikro-Western blot Verfahren

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Summary:

Herpes simplex virus type 2 (HSV-2) is the main cause of herpes genitalis, a recurrent sexually transmitted disease. By the use of routine serologic methods (complement fixation test, enzyme immunoassay), virus carriers are difficult to identify because of strong antibody cross reactions with antigens of HSV-1, which is ubiquitously spread throughout the population. We introduce a microtechnique Western blot system loaded with HSV-1 and HSV-2 type-specific and common antigens on separated nitrocellulose strips. By the simultaneous evaluation of immunologic reactions with both strips, the occurrence of HSV-2 specific antibodies can be sensitively detected in serum specimens containing antibodies to HSV-1. A total of 158 serum specimens were analyzed and the results obtained by Western blot were compared to those of a screening ELISA and virus isolation performed with smears of herpes lesions.

An agreement of 97.9 % was assessed between Western blot and virus isolation to detect an HSV-1 and HSV-2 infection. Less specific serologic results were produced by the screening ELISA on HSV-2 antibodies which correlated in 85.4 % (41/48) with virus isolation and typing. Concerning HSV-2 antibody testing, Western blot and ELISA showed an overall agreement in 89.8 % of the sera investigated.

As shown by our data, the HSV type specific Western blot proved to be a specific, reproducible and standardized technique. It can be utilized for both sero-epidemiological surveys and determination of the HSV immune status.

Keywords:

Western blot – HSV type specific antibodies – virus isolation – screening ELISA

Zusammenfassung:

Herpes simplex Virus Typ 2 (HSV-2) ist die häufigste Ursache für Herpes genitalis, eine sexuell übertragbare und häufig rekurrierende Erkrankung. Mit den routinemäßig eingesetzten serologischen Methoden (Komplementbindungsreaktion, Enzymimmunoassay) sind auf Grund der hohen Kreuzreaktivität mit dem ubiquitär verbreiteten HSV-1, Virusträger nur schwer identifizierbar. In der vorliegenden Arbeit wird ein Mikro-Western blot System vorgestellt, beruhend auf mit HSV-1 und HSV-2 typen- und gruppenspezifischen Antigenen bestückten Nitrozellulosestreifen. Auf Grund der Reaktivität mit typenspezifischen Proteinbanden ist der Nachweis von HSV-2-Antikörpern in Serumproben, welche zusätzlich HSV-1-Antikörper enthalten, möglich.

Insgesamt wurden 158 Serumproben untersucht und die Western-blot-Ergebnisse mit denen eines Screening-ELISA und der Virusisolierung aus Herpes Läsionen verglichen.

Western blot und Virusisolierung stimmten bei 97,9 % der untersuchten Proben überein. Weniger spezifische Ergebnisse wurden mit dem ELISA erzielt, welcher nur in 85,4 % (41/48) der Fälle mit der Virusisolierung übereinstimmte. Western blot und ELISA korrelierten für die HSV-2-Serotypisierung bei 89,8 % der untersuchten Proben.

Der Western blot erwies sich als ein spezifisches, reproduzierbares und standardisierbares Verfahren und kann sowohl für seroepidemiologische Durchseuchungsstudien als auch zur Festlegung des Immunstatus eingesetzt werden.

Schlüsselwörter:

Western blot – HSV-typenspezifische Antikörper – Virusisolierung – Screening-ELISA

Introduction

Herpes simplex viruses (HSV) type 1 and 2 are widspread human infectious agents and are responsible for persistent and latent infections. HSV-1 infection is commonly acquired during childhood without clinical signs. However in 10%, primary infection or subsequent recurrences are associated with oral skin lesions or rare complications (retinitis, encephalitis). Infections with HSV-2 causing recurrent herpes genitalis are usually acquired through sexual contact.

Detection of type-specific HSV antibody is important for more than one reason. Epidemiologic surveys showed, that about 86% of genital herpes simplex infections are caused by HSV-2 and only about 14% by HSV-1 (1). HSV-2 infections of the genitals recur four times as often as HSV-1 infections (2), and are characterized by longer duration and shorter asymptomatic intervals (3). Furthermore, life-threatening generalized neonatal herpes is mainly caused by HSV-2.

HSV-2 antibody typing is necessary for identifying pregnancies likely to be complicated by recurrent maternal HSV-2 infection. Serologic surveys concerning the prevalence and incidence of HSV-2 in population groups at increased risk of human immunodeficiency virus (HIV) infection may provide information concerning changes in the sexual behaviour of these groups and identify those patients at risk of recurrent genital herpes.

Laboratory diagnosis of HSV-2 infection is performed by virus isolation in various cell cultures (4) or antigen detection by immunofluorescence or ELISA (5, 6).

Detection of HSV antibodies is the most specific technique for diagnosing past HSV infection and for seroepidemiologic surveys. Cross-reacting antibodies common to both serotypes rendered type specific serologic diagnosis difficult. For statistical evaluations the prevalence of HSV 2 infected persons can be evaluated by calculating the ratio of antibodies against HSV 1 and 2 (7, 8). Thus, methods of antibody differentiation between HSV type 1 and 2 were applied mainly to epidemiologic surveys (7) and not to individual examination or prophylactic screenings during pregnancy.

In this study, a microtechnique Western blot for HSV type specific antibody detection is described and compared to virus isolation and a screening ELISA (7) previously introduced. Serum samples obtained from different clinical groups were investigated and the results obtained by Western blot were compared to antigen typing in patients from whom swab samples were obtained. Furthermore the epidemiologic plausibility of the serologic tests (ELISA, Western blot) was investigated in groups of patients at different risk of HSV-2 infection.

Materials and methods

Patient specimes

A total of 158 serum specimens which had been previously tested by a commercialy available ELISA for the detection of HSV-antibodies (Enzygnost, Behringwerke, Marburg) were obtained from patients attending the University Clinics of Frankfurt a. M. from 1989 to 1990. They included 68 males. 68 females and 22 prepuberty children.

The patients were assigned 6 different groups which were defined as follows:

Group 1: 38 patients from whom HSV-2 strains were isolated from primary or recurrent genital herpes lesions. All the 38 sera were obtained at variable intervals after occurrence of herpes lesions (certainly HSV-2 positive).

Group 2: 10 patients from whom HSV-1 was isolated from recurrent oro-labial herpes (certainly HSV-1 positive).

Group 3: 22 HSV seropositive children aged from 2 to 8 years (probably only HSV-1 positive, if no sexual assault is suspected).

Group 4: Included in this group were 20 female prostitutes registered at the public health office of Frankfurt/Main (elevated risk for HSV-2).

Group 5: 58 serum samples which were randomly selected from the routine serologic diagnostic of the Department of Medical Virology (considered as clinically normal persons without known risk factors for HSV-2 infection).

Group 6: included 10 anti-HSV seronegative persons aged from 16 to 24 years.

Clinical specimens for virus isolation were obtained by scraping skin vesicles and were transferred in modified Stuart medium (Culturette™, Becton Dickinson, Heidelberg, Germany). Serum samples were stored at -20°C until tested.

Screening ELISA on HSV serum antibodies

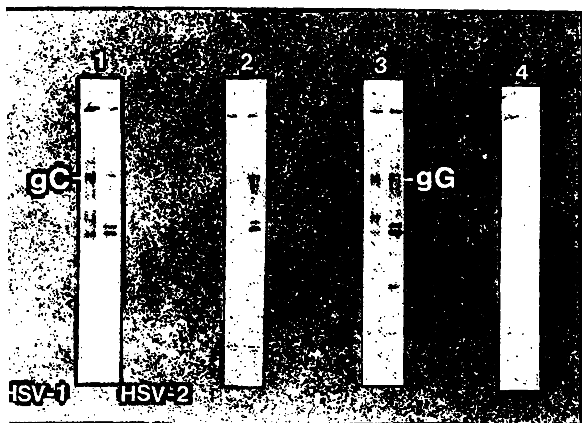
Serum antibodies to HSV-1 and HSV-2 antigen were determined according to the method described by Dannenmaier et al. (7). Briefly, detection of serum antibodies against carrier fixed capsid antigens of HSV-1 and HSV-2 was performed. Similar to previous aproaches in the statistical evaluation of type-specific neutralizing antibodies of HSV type 1 and 2 in human sera (8), we calculated the ratio of ELISA antibody values HSV-2/HSV-1.

Western blot

Reference strains of HSV-1 (Mc Intyre, ATCC) and HSV-2 (MS, ATCC) were propagated on RC-37 Rita cells (monkey kidney cells). A confluent cell layer ($3x10^7$ cells/Roux flask) was infected at a MOI of 0,1. After an incubation time of 3 days at 37°C, approximately 90% of the cells showed virus specific CPE. Cells were frozen and thawed 3 times. Low-speed centrifugation (2,400 g, 10 min, 4°C) was used to separate the cell debris. The supernatant was concentrated by ultracentrifugation (40,000 g, 12 h, 4°C) and the residual pellet was resuspended in 1 ml minimal essential medium (MEM). Mock infected cells were prepared using the same procedure.

SDS-PAGE was performed using the PhastSystem™ (Pharmacia, Uppsala, Sweden) on Phast-Gel 8–25% gradient gels. Crude viral antigens of HSV-1 and HSV-2 were suspended in sodium dodecyl sulphate (SDS) sample buffer (0.01M Tris/HC1, pH 8.0, 0.001M EDTA; 1% mercaptoethanol, 2% SDS) and boiled at 100°C for 5 min. Marker proteins (Pharmacia, Uppsala, Sweden) were separated along with the viral antigens and were visualized by silver nitrate staining (Fig. 2) in order to permit protein identification by their approximative molecular weights.

The Western blot was performed according to the method described by Braun and Abraham (9). Briefly, a polyvinylidene difluoride (PVDF) membrane (Immobilon™) was used as blotting membrane (pore size: 0.5 μm; protein binding capacity: 190 $\mu g/cm^2$). The membrane was equilibrated in methanol/H_2O (90% v/v) for 5 min, follo-



g. 1: Western blot analysis of 4 patient sera (left lane HSV-1, ght lane HSV-2). The type-specific bands gC (HSV-1) and gG SV-2) are prominent. The week reaction on the gC-band in line might be an indicator for an additional HSV-1 infection with a w antibody titer

HSV-1-positive serum (type-specific band of HSV-1 gC)

HSV-2-positive serum (type-specific band of HSV-2 gG)

serum with antibodies against both serotypes

HSV-negative serum

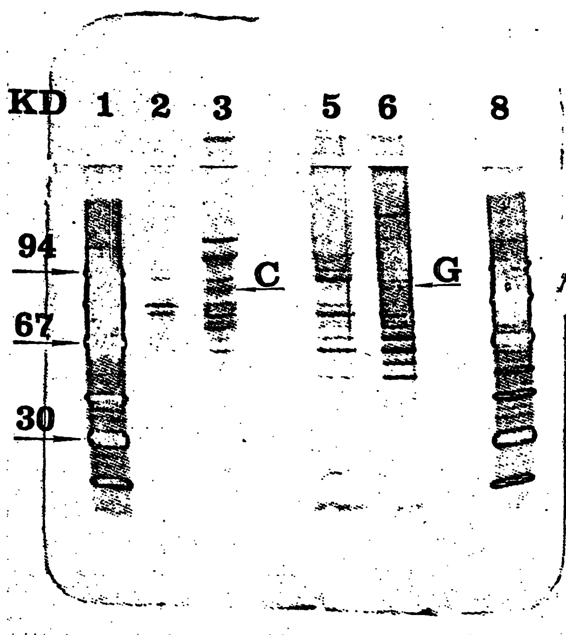


Fig. 2: SDS-PAGE analysis of HSV type 1 and type 2 antigen: Marker proteins in lanes 1 and 8. Type-specific bands of HSV-1, gC, (lane 3) and HSV-2, gG, (lanes 5 + 6) are clearly recognisable. Lane 2 shows cell proteins of uninfected RC-37 Rita cells.

ed by 30 min in blotting buffer (0.4M glycin; 10% me-anol; 0.1% SDS; 50.0mM Tris; in aqua bidest.) prior to otting.

ne blot strips were stored at -70°C or used directly for e Western blot. After 30 min blocking in blocking solu-on (BS, BSA 2%, goat serum 5%, NaCL 0.2M, Thimero-l 0.01%, Tris 0.02mM, Tween 20, 0.2%, pH 7.45) follo-ed by application of the sera (1:50 in BS) and incuba-on for 12h at 4°C, the strips were washed three times) min each) with washing buffer. In subsequent steps, e mouse monoclonal anti-human IgG antibodies (Dia-ova, Hamburg, FRG) (1:1,000 in BS) were applied, as ell as biotinylated monoclonal goat anti-mouse kappa-ain antibodies (Dianova, Hamburg, FRG) (1:1,000 in S) and streptavidin peroxidase (1:2,000 in BS), each for h at room temperature. In the final washing step, ace-te buffer (0.05M sodiumacetate, pH 5) was used for an ditional 10 min rinse. Reactive bands were visualised addition of amino-ethyl-carbazol (AEC) (105 ml acetate ffer; 0.18 g AEC; 45 ml N-N dimethylformamid) plus % H_2O_2. The colour reaction was stopped with a single ua bidest. rinse.

serum sample was considered positive for HSV-1 speci-antibody if a reactive band corresponding to the gC is detected. Alternatively, a reactive band correspon-ng to the gG protein was indicative for the presence of tibody against HSV-2.

us Isolation Procedure

nical specimens were resuspended in MEM containing nicillin, streptomycin and amphotericin and were ino-lated to primary amnion cell cultures up to six hours er sampling. After the development of a typical cytopa-ogenic effect (CPE), the positive isolates were subjec-d to antigen typing with a commercially available im-unofluorescence test (IFT) (Syva, Palo Alto, USA), using onoclonal antibodies. The samples were processed ac-rding to the manufacturer's instructions.

Results

Western blot analysis

As shown in figure 1, the Western blot detected antibodies against type-specific glycoproteins of HSV-1 and HSV-2, gC (gC-1) and gG (gG-2). Homologous reactions were characterised by the presence of a significantly increased number of specific bands as compared to heterologous reactions.

Diffuse bands produced by gC (HSV-1) and gG (HSV-2) as shown by silver staining were of approximative molecular weights of 92,000 Dalton (Fig.2). A sharp band visualized above the gC and gG bands (lane 3) was attributed to a cellular protein as shown by SDS-Page analysis of mock infected cells (lane 2).

To verify the identity of the gC and gG bands, blots were reacted with 2 different monoclonal antibodies directed against gC and gG (Camon, Wiesbaden, FRG). Banding patterns corresponding to the diffuse 92 kD protein bands of gC and gG were detected (data not shown).

The specificity of the Western blot method was determined using a panel of sera which were HSV-antibody negative, but positive for IgG-antibodies against different viruses of the herpes virus family, including cytomegalovirus (CMV), Epstein-Barr virus (EBV) and varicella zoster virus (VZV). None of the HSV-IgG negative control sera reacted in the Western blot indicating its high specificity. Faint background bands were seen when HSV positive sera reacted with proteins from mock-infected cells. Mouse monoclonal antibody to human IgG was deleted from the assay, and no reactive protein bands were detected, indicating that the biotin labelled rat anti-mouse IgG conjugate did not cross react with human IgG.

Tab. 1: Comparison of type specific antibody detection by Western blot and ELISA in 158 sera obtained from patient groups at different risk of HSV-2 infection

patient group	Western blot				ELISA		
	HSV-1 pos.	HSV-2 pos.	HSV-1 and HSV-2 pos.	neg.	HSV-1 pos.	HSV-2 pos.	neg.
Group 1 (n = 38) HSV-2 isolated in cell culture	1 (2.6%)	7 (18.4%)	30 (80%)	0 (0%)	5 (13.2%)	33 (86.8%)	0 (0%)
Group 2 (n = 10) HSV-1 isolated in cell culture	10 (100%)	0 (0%)	0 (0%)	0 (0%)	8 (80%)	2 (20%)	0 (0%)
Group 3 (n = 22) prepuberty children	22 (100%)	0 (0%)	0 (0%)	0 (0%)	22 (100%)	0 (0%)	0 (0%)
Group 4 (n = 20) prostitutes	6 (30%)	5 (25%)	8 (40%)	1 (5%)	7 (35%)	13 (65%)	0 (0%)
Group 5 (n = 58) no known risk factor	36 (62,1%)	3 (5.2%)	6 (10.3%)	13 (22.4%)	34 (58.6%)	9 (15.5%)	15 (25.9%)
Group 6 (n = 10) HSV seronegative individuals	0 (0%)	0 (0%)	0 (0%)	10 (100%)	0 (0%)	0 (0%)	10 (100%)

Of the 158 sera investigated, antibody only to HSV-2 was detected in 15 sera by Western blot. Seventy-five sera presented antibody to HSV-1 and 44 sera were seropositive for both HSV-1 and HSV-2.

HSV-2 bands were faint when reacted with sera containing antibodies to HSV-1 only. Sera with both HSV-1 and HSV-2 antibodies presented full antibody profiles on both HSV-1 and HSV-2 strips.

Comparison of ELISA, Western blot and virus isolation

The investigations performed with 158 sera showed a high degree of correlation between results obtained with serological tests (Western blot, ELISA) and those of virus isolation. Concerning HSV-2 antibody detection, ELISA and Western blot matched in 89.8% of the sera. Our data indicate that virus isolation combined to antigen typing by IFT verified the reliability of both Western blot and ELISA (table 1). In 47 (97.9%) patients, HSV antibody typing by Western and HSV antigen determination by IFT showed concordant results. In one serum sample obtained from a patient in the early phase of primary HSV-2 infection, the Western blot gave a negative result. Virus culture and ELISA results matched in 85.4% (41 of 48 sera).

Correlation of Western blot and ELISA on HSV-2 antibodies in patient groups at different risk of HSV infection

The comparative results obtained by Western blot and ELISA in 6 patient groups at different risk are depicted in table 1. Out of 38 patients with positive HSV-2 typing, 37 and 33 were considered HSV-2 positive by Western blot and ELISA respectively. ELISA showed 2 putative false positive results in patients with positive culture for HSV-1, whereas Western blot concorded with virus isolation and antigen typing in all the 10 patients investigated. In prepuberty children with no risk of exposure to HSV-2 both Western blot and ELISA were negative. A total of 13 (65%) prostitutes were tested HSV-2 positive by both Western blot and ELISA. Out of 58 serum samples (group 5), 9 (15.5%) were classified as positive in both assays. Ten serum specimens obtained from persons known to be HSV seronegative showed no antibodies against HSV-1 or HSV-2 as well in the Western blot and in the ELISA.

Discussion

In the present study, a microtechnique Western blot for type specific HSV antibody detection is described and compared to virus isolation with subsequent antigen typing by IFT. The test system yields highly reproducible results as compared to reference methods, e. g. virus isolation and typing. Furthermore, no cross reactivities with sera obtained from patients with acute infections caused by other herpes viruses than HSV (CMV, EBV, VZV) were observed. These results are in agreement with those of other authors which used conventional Western blot systems (9, 10).

In contrast to other authors, glycoprotein C (gC) of HSV-1 showed a molecular weight of 92 kD. Modifications of the electrophoretic mobility attributable to the Phast gel™ system, i. e. introduction of negatively charged groups or chemical blocking by positive charges (11) may be responsible for the discrepancy in molecular weight. Apparent molecular weight may not be a reliable marker to identify viral proteins, especially when different electrophoretic systems are compared (12). As an alternative, glycoproteins gC and gG were identified by monoclonal antibodies. This study provided evidence for the excellent ability of the Western blot to differentiate HSV type specific antibodies. The concordance between Western blot and virus isolation with antigen typing by IFT was 97.7 %. In the 158 sera obtained from individuals at different risk of HSV infection, the overall agreement between Western blot and ELISA was 89.8% thus corresponding to data obtained by previous authors (13).

Concerning the epidemiologic plausibility of both Western blot and ELISA, the ELISA assay produced a higher rate of false positive and false negative results as compared to Western blot and to virus isolation combined to antigen typing by indirect immunofluorescence staining. Although the Western blot is more labour intensive, it is

suited for seroepidemiologic surveys since it showed a good correlation with antigen typing.

Immunoblot analysis for the differentiation of type specific HSV antibodies with standardized techniques employing an integrated electrophoresis system for the separation of viral proteins, represents a simple and rapid method for the large scale production of nearby identical blot strips thus enabling routine testing with reproducible results. It can be utilized for both determination of the HSV immune status or for epidemiological surveys of HSV-1 or HSV-2 infections.

Recently type specific recombinant HSV antigens (gC and gG) are available for serologic assays. Nevertheless, the production of those antigens remains expensive, and problems with sensitivity might occur. The broad spectrum of viral antigens recognized by polyclonal immuneresponses in vivo can be visualized by more sophisticated assays, like radioimmunoprecipitation or Western blots (13, 14).

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