ANTIBODY RESPONSES TO THE HERPES SIMPLEX VIRUS TYPE 2 GLYCOPROTEIN G IN SERA OF HUMAN IMMUNODEFICIENCY VIRUS-INFECTED PATIENTS IN SLOVAKIA

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Summary. – Thirty sera of human immunodeficiency virus-positive (HIV+) and 37 sera of HIV-negative (HIV-) individuals in Slovakia were tested for the presence of antibodies to herpes simplex virus type 2 (HSV-2) glycoprotein G (gG). A notable difference between the prevalence of HSV-2-specific antibodies in HIV+ and that in HIV- individuals was found (37% vs. 11%) confirming and extending previous reports that HSV-2 infection is an important risk factor for HIV transmission. Efforts toward the detection of HSV-2 infection and its therapy by anti-HSV drugs should be considered an important factor in decreasing the risk of contracting and spreading of HIV in Slovakia.

Key words: herpes simplex virus type 2; human immunodeficiency virus; glycoprotein G-2; immunoblot analysis; ELISA; antibodies; human sera

Introduction

Relatively early at the beginning of the acquired immunodeficiency syndrome (AIDS) epidemic a close link between sexually transmitted diseases manifested by genital ulcers and HIV infection has been noticed (Wasserheit 1992; Holmberg *et al.*, 1988; Halbert *et al.*, 1986). HSV-2 infection causing genital ulceration is one of the most common opportunistic infections in HIV-infected patients. A disruption of normal epithelial barriers of the genital organs caused by such ulcerative infections can facilitate HIV transmission. Although the majority of HSV-2 infections may go inapparent (Fleming *et al.*, 1997; Koutsky *et al.*, 1990), asymptomatically infected persons can systematically spread HSV-2 infection and increase risk of HIV infection (Forsgren *et al.*, 1994; Langenberg *et al.*, 1989). Accumulating epidemiological and serological evidence indicates that HSV-2 infection may be an important risk factor influencing HIV infection (Griffiths 1998; Hook *et al.*, 1992; Holmberg *et al.*, 1988).

An HSV-2 infection can be confirmed by testing the relevant serum for the presence of antibodies to HSV gG-2. Since HSV-2 but not HSV-1 can induce antibody response to gG-2, the detection of gG-2-specific antibodies in human serum provides reliable evidence of previous exposure to HSV-2 infection (Liljeqvist *et al.*, 1998; Groen *et al.*, 1998; Ho *et al.*, 1993). Recently, we have described preparation of monoclonal antibodies (MoAbs) specific for various HSV glycoproteins including MoAb specific for gG-2 (Bystrická *et al.*, 1991).

Abbreviations: AIDS = acquired immunodeficiency syndrome; BSA = bovine serum albumin; DAS-ELISA = double-antibody sandwich enzyme-linked immunosorbent assay; gB = glycoprotein B; gG = glycoprotein G; HIV = human immunodeficiency virus; HIV+ = HIV-positive; HIV- = HIV-negative; HSV-2 = herpes simplex virus type 2; MoAb = monoclonal antibody; OCG = octyl b-D-glucopyranoside; PBS = phosphate-buffered saline; PMSF = phenylmethylsulfonyl fluoride; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

In this report, we describe development of an accurate gG-2-specific double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and immunoblot test with purified gG-2 for assessment of antibodies to gG-2 in sera of tested individuals. We found that the occurrence of the antibodies to gG-2 was considerably higher in HIV+ (37%) than in healthy (HIV–) individuals (11%). We suggest the that genital herpes is an important factor of spreading of HIV in Slovakia.

Materials and Methods

Cells, viruses and antigen extracts. Vero cells were propagated and maintained as described previously (Bystrická *et al.*, 1991). HSV-2 strain Praha was obtained from Prof. V. Vonka, SEVAC, Prague, Czech Republic. For preparation of antigen extracts, monolayers of Vero cells were infected with HSV-2 at the multiplicity of 1 – 5 PFU/cell for 24 – 48 hrs. When the infected cells showed a 70% CPE, they were washed with cold phosphate-buffered saline (PBS), scraped off the glass and pelleted. The pellet was solubilised with an extraction buffer (0.5 mol/l NaCl containing 30 mmol/l n-octyl-β-D-glucopyranoside (OCG) and a cocktail of proteinase inhibitors (Complete, Boehringer)) for 30 mins on ice. The cell debris was sedimented at 1,500 x g for 6 mins and the supernatant was collected and stored in aliquots at -20°C. An extract of non-infected cells was prepared in the same way.

MoAbs. MoAb 303 to HSV gG-2 and MoAb 499 to HSV gB-2, and type-common MoAbs T111 and 170 to HSV gB-1,2 were used (Bystrická *et al.*, 1991).

Human sera. Thirty human sera of HIV+ patients were collected at the National Reference Centre for Prevention of AIDS/HIV, Bratislava. The presence of antibodies to HIV in these sera was demonstrated for each patient by analysing two independent serum samples first by an immunoassay (Abbott Laboratories or Sanofi Diagnostics Pasteur) and later by immunoblot assay (HIV Western Blot, Murex). Clinical characteristics of the patients (representing both sexes) regarding their history of genital or anogenital herpes and risk factors for contracting HIV are shown in Table 1. Thirty-seven sera from randomly selected healthy donors were collected at the Ružinov Hospital in Bratislava and tested for the presence of antibodies to HIV. The sera were considered HIV-negative by standard ELISA for detection of HIV antibodies (Abbot Laboratories or Sanofi Diagnostics Pasteur).

One positive and one negative human serum were used throughout the study as reference for the presence or absence of antibodies to HSV-2. A positive reference serum containing high level of antibodies to HSV gG-2 was collected from a patient suffering from reccurent genital herpes for many years.

DAS-ELISA. For quantitation of antibodies to HSV gG-2 and gB-1,2 we used DAS-ELISA described by Hashido *et al.* (1997). A checkerboard titration was performed to determine optimal dilutions of ascites fluids containing MoAbs, extracts of HSV-2-infected cells and anti-human conjugate. Duplicate wells of flat-bottom 96-well KOOH-I-NOOR microplates were coated with 50 µl of anti-gG-2 ascites fluid (MoAb 303) or anti-gB-1,2 ascites fluid (mixture prepared from equal volumes of MoAbs 499, T111, and 170) diluted 1 1,000 or 1:4,000 in PBS and incubated overnight at +4°C. The plates were washed once with a the washing buffer (PBS containing 0.02% Tween) and the non-specific binding was blocked with 200 µl of PBS containing 2% non-fat dry milk for 2 hrs at room temperature. After washing the plates 3 times with the washing buffer, 50 µl of the extract of HSV-2-infected or non-infected Vero cells diluted 1:60 for gG-2 assay and 1:160 for gB-1,2 assay in PBS containing 0.1% bovine serum albumin (BSA) was dispensed into wells. The plates were incubated overnight at 4°C and washed 4 times with the washing buffer. Fourfold dilutions of human sera (from 1:100 to 1:400,000) were prepared in 0.5% BSA in PBS and 50 ml of each dilution was dispensed in duplicate wells. The plates were incubated for 2 hrs at 37 °C and washed 4 times with the washing buffer. Then 50 µl of peroxidase-conjugated antibodies to human globulins (Dako, Denmark) diluted 1: 5,000 in 1% BSA in PBS was added to each well and the plates were incubated for 2 hrs at 37°C. After fourfold washing, 50 µl per well of 1 mg/ml o-phenylenediamine in 0.1 mol/l citrate buffer pH 5.0 containing 0.03% H_2O_2 was added. After 7 mins of development in the dark, the colour reaction was terminated by addition of 50 µl of 2N H_2SO_4 to each well. Before reading the absorbance at 492 nm in an ELISA autoreader (Labsystems MR), the plates were carefully shaken to disperse the coloured product over the bottom of the wells. The background A_{492} value was obtained by treating 12 wells in the microtitre plate exactly as above except for the diluted human sera were replaced by the diluent.

The titre of the antibodies was defined as the reciprocal of the highest serum dilution at which the A_{492} value was at least a threefold of that of the reference HSV-negative serum at the same dilution.

Purification of HSV gG-2 was performed by immunoaffinity chromatography. An affinity column was made with 6 mg of purified MoAb 303 and 1 g of CNBr-Sepharose (Pharmacia) according to instructions of the manufacturer. The solubilised membranes of HSV-2 infected cells were prepared from about 350 x 10^6 Vero cells washed once with cold PBS and then extracted with an extraction buffer (10 mmol/l Tris pH 7.0, 0.5 mol/l NaCl, 0.5% sodium deoxycholate, 0.5% OCG and a cocktail of proteinase inhibitors in concentrations recommended by the manufacturer (Complete, Boehringer)). The extraction lasted for 30 mins on ice and then the mixture was clarified for 20 mins at 1,500 x g. The supernatant was centifuged at 15,000 rpm (Beckman, rotor 20) for 30 mins. The supernatant was added to CNBr-Sepharose coupled with purified MoAb 303 and incubated overnight at 4°C under constant shaking. The gel (5 ml) was centrifuged at 600 x g for 2 mins and washed at least 5 times with 300 ml of a washing buffer (0.01 mol/l Tris pH 7.5, 0.5 mol/l NaCl, 0 05% OCG and 0.1 mmol/l phenylmethylsulfonyl fluoride (PMSF)). gG-2 was eluted with 5 ml of cold 3 mol/l KSCN in 0.01 mol/l Tris pH 7.8 for 15 mins under constant shaking. The elution was repeated twice under the same conditions. The pooled eluates containing gG-2 were immediately dialysed against PBS for 3 days with 3 – 4 changes of the buffer. The final product showing two major bands in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) corresponding to

different glycosylation forms of gG-2 was used as the antigen in the immunoblot test for detection of antibodies to gG-2. However, the purity of this product was not satisfactory enough for reliable detection of these gG-2-specific antibodies by sensitive ELISA.

Immunoblot analysis. The whole procedure except the incubation with a second antibody has been described in detail elsewhere (Bystrická *et al.*, 1991). Briefly, 0.6 ml of purified gG-2 in PBS with an approximate protein concentration of 0.05 mg/ml was electrophoresed by standard SDS-PAGE in 8.5% gel. After the run, the gel was equilibrated with a transfer buffer (25 mmol/l Tris, 192 mmol/l glycine and 10% methanol) for 1 hr and electroblotted to a nitrocellulose membrane (Schleicher and Schuell, 0.22 μm) in a Mini Trans-blot cell (BioRad) using parameters recommended by the manufacturer. Then the remaining nitrocellulose binding sites were blocked by incubating the membrane in PBS containing 5% non-fat dry milk for 60 mins. The nitrocellulose membrane was cut into strips and incubated with human sera diluted 1:100 or 1:200 in PBS containig 1% non-fat dry milk for 2 hrs with constant agitation. The detection of the antibody bound to the blot was accomplished by incubation of strips with an anti-human (Dako, Denmark) or anti-mouse (SEVAC, Czech Republic) conjugate diluted 1:1000 in PBS containing 1% non-fat dry milk for 1 hr at room temperature followed by visualisation with 0.06% 3,3-diaminobenzidine tetrahydrochloride in 0.05 mol/l Tris-HCl pH 7.6 containing 0.03% H_2O_2 and 0.03 % $CoCl_2$

Results

Detection of antibodies to HSV gG-2 and gB-1,2 by DAS-ELISA

Eleven (Nos. 11, 12, 16, 17, 19, 22, 26, 37, 45, 56, and 80) of 30 HIV+ patients showed medium (6,400) to high (25,000) titres of serum antibodies to gG-2 (Table 1). A low level of antibodies was detected in sera of 9 patients (titres ranging from 1,600 to 3,200), but their anti-gG-2 specificity was not confirmed by immunoblot analysis. The remaining sera of 10 patients were negative (titres < 1,600) in this test.

Four of 37 HIV- sera had medium (6,400) to high (25,000) titres. Three sera with titres of 6,400 were included into another group containing 14 sera with lower titres because the immunoblot analysis did not confirm their anti-gG-2 specificity. The remaining 19 sera were DAS-ELISA-negative (titres < 1,600) (Table 2).

To verify that the patients with antibodies to gG-2 were actually exposed to HSV infection, antibodies to HSV gB-1,2 were also estimated. Because HSV immune sera usually show highest titres of antibodies specific for this particular HSV glycoprotein (Eberle and Mou 1983), we selected antibodies to gB-1,2. There was a good correlation between the titres of antibodies to gG-2 and those to gB-1,2. Most of the HIV+ as well as HIV- sera with medium or high titres of antibodies to gG-2 also showed rather high titres (1:25,000 to 1:100,000) of antibodies to gB-1,2 (Tables 1 and 2). A demonstration of antibodies to gB-1,2 in sera containing also antibodies to gG-2 proves HSV infection in corresponding patients but does not differentiate between HSV-1 and HSV-2. In conclusion, the DAS-ELISA results suggest that HSV-2 is more frequently detected in HIV+ than in HIV- sera.

For preparation of infected cell extracts used in DAS-ELISA we solubilised cells by mild detergent OCG. There is no guarantee that the solubilisation of cells under these conditions allowed complete dissociation of all protein

Table 1. Clinical characteristics of HIV+ patients DAS-ELISA titres and immunoblot reactivity of their sera

Patient No.	Gender	Infection with HIV	History of genital HSV	Titre of antibodies to gG-2[a]	Titre of antibodies to gB-1,2[a]	Antibodies to gG-2[b]
11	M	Homo	None	12,000	25,000	+
12	M	Homo	None	6,400	6,400	+
16	M	Homo	Recurrent	6,400	6,400	+
17	M	Homo	Recurrent	6,400	6,400	+
19	M	Homo	Recurrent	6,400	10,000	+
22	M	Homo	None	6,400	6,400	+
26	M	Homo	None	6,400	15,000	+
37	M	Homo	Recurrent	20,000	25,000	+
45	F	Hetero	None	25,000	25,000	+
56	M	Homo	Recurrent[c]	12,000	25,000	+
80	F	Drug	Unknown	12,000	50,000	+
1	F	Hetero	None	1,600	20,000	–
30	M	Homo	None	3,200	25,000	–
32	M	Homo	None	1,600	6,400	–
34	M	Homo	None	1,600	6,400	–
35	M	Homo	None	1,600	1,600	–
43	M	Homo	None	1,600	1,600	–
53	M	Homo	None	1,600	<1,600	–
54	F	Hetero	None	1,600	1,600	–
55	M	Hetero	None	1,600	1,600	–
14	M	Homo	Unknown	<1,600	<1,600	–
15	M	Transf.	None	<1,600	<1,600	–
20	M	Homo	None	<1,600	<1,600	–
39	M	Homo	None	<1,600	6,400	–
42	M	Homo	None	<1,600	6,400	–
51	M	Drug	None	<1,600	<1,600	–
52	M	Homo	None	<1,600	6,400	–
57	M	Homo	None	<1,600	1,600	–
85	M	Homo	Unknown	<1,600	<1,600	–
97	M	Unknown	Unknown	<1,600	<1,600	–
Positive reference serum			Recurrent	25,000	100,000	+
Negative reference serum			None	1,600	<1,600	–

[a]DAS-ELISA.
[b]Immunoblot analysis.
[c]Outbreaks of the disease were reported last year.
(+), (–) = present, absent. Homo, hetero = homosexual, heterosexual orientation of the patient. Drug = i.v. drug user. M,F = male, female. Transf. = patients that contracted HIV during blood transfusion outside Slovakia.

Table 2. DAS-ELISA titres and immunoblot reactivity of HIV– sera

No. of patients in the group	Titre of antibodies to gG-2[a]	Titer of antibodies to gB-2[a]	Antibodies to gG-2[b]
4	6,400 – 25,000	12,500 – 100,000	+
14	1,600 – 6,400[c]	<1,600 – 50,000	–
19	< 1,600	<1,600 – 25,000	–
Positive reference serum	25,000	100,000	+
Negative reference serum	<1,600	<1,600	–

[a]DAS-ELISA.
[b]Immunoblot analysis.

complexes. Provided that a part of gG-2 remained in the complex with other viral glycoproteins, e.g. gB-1,2, the DAS-ELISA titres of antibodies specific for gG-2 were overestimated. Furthermore, we are aware of the fact that our DAS-ELISA results have been affected by the selection of the particular negative control serum and by choosing the value distinguishing negative from positive titres. Consequently, low titres of gG-2-specific antibodies in an extremely sensitive DAS-ELISA may be falsely interpreted as an evidence for HSV-2 infection. In order to verify the DAS-ELISA data and confirm HSV-2 infection, we detected gG-2-specific antibodies in human sera also by immunoblot analysis.

Detection of HSV gG-2-specific antibodies by immunoblot analysis

Initially, we were using extracts of infected cells for detection of gG-2-specific antibodies by immunoblot analysis. The resulting patterns, however, were extremely complex and an accurate identification of gG-2 bands on test strips was problematic (Ashley *et al.*, 1988). An easy and unequivocal identification of antibodies to gG-2 was achieved by replacing extracts of HSV-2-infected cells by purified gG-2 in all experiments described here.

With MoAb 303 specific for gG-2 we detected 2 bands with apparent M_r of about 90 K and 70 K (Fig. 1, lanes D, E). These 2 bands apparently corresponded to different glycosylation forms of gG-2 (Liljequist *et al.*, 1998; Ashley *et al.*, 1988). We regarded the appearance of these bands as an unambiguous evidence for the presence of gG-2-specific antibodies in examined sera. In some cases, other distinct bands were also visible; they might represent either differently processed or degraded forms of gG-2 (lanes B, C, F, G, H, I) or some contamination.

The representative results obtained with sera of HIV+ patients Nos. 17 and 45 (lanes F,G,H,I), and the reference human serum positive for gG-2-specific antibodies

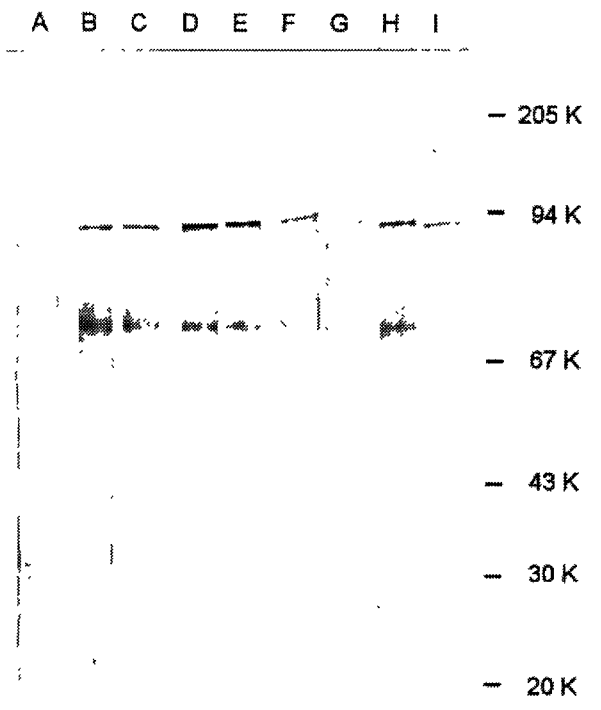

Fig. 1
Immunoblot analysis of MoAb 303 and human sera
Purified gG-2 was subjected to SDS-PAGE, blotted and probed with MoAb 303 and human sera. M_r values of size markers are indicated on the right. Negative reference human serum diluted 1:100 (lane A), positive reference human serum diluted 1:100 and 1:200 (lanes B,C), MoAb 303 as ascites fluid diluted 1:1000 and 1 2000 (lanes D,E), HIV+ human serum No. 17 diluted 1.100 and 1:200 (lanes F,G), HIV+ human serum No. 45 diluted 1:100 and 1:200 (lanes H,I).

(lanes B,C) are also shown in Fig. 1. It is evident that the sera Nos. 17 and 45, and the positive reference serum gave the same pattern as the mouse MoAb 303 specific for gG-2, confirming the presence of gG-2-specific antibodies in these sera. The negative reference human serum failed to react with purified gG-2 (lane A). These preliminary experiments clearly showed that the immunoblot test was accurate and gG-2-specific.

Results of the immunoblot analysis are summarized in Tables 1 and 2. In the HIV+ group, all sera with DAS-ELISA titres of gG-2 antibodies higher or equal to 6,400 (Nos 11, 12, 16, 17, 19, 22, 26, 37, 45, 56, and 80) were found positive, and all sera with DAS-ELISA titres lower or equal to 3,200 were found negative. In the group of 37 HIV– sera, 4 donors with DAS-ELISA titres of gG-2 antibodies higher or equal to 6,400 were found positive also in immunoblot analysis. Although all 6 HIV+ sera with DAS-ELISA titres equal to 1:6,400 were also immunoblot-positive, only 1 of

4 HIV– sera with the same DAS-ELISA titre showed distinct gG-2-specific bands. These results suggested that there was no correlation between the DAS-ELISA titre and the outcome of the immunoblot test. Consequently, for the 3 HIV donors with DAS-ELISA gG-2 titres equal to 6,400 but immunoblot-negative we have no good evidence for HSV-2 infection. None of the sera tested in both groups of the patients was immunoblot-positive and DAS-ELISA negative.

Taken together, the immunoblot analysis complemented and verified DAS-ELISA results and strengthened the idea that HSV-2 infection was more common in HIV+ than in HIV– individuals.

Clinical manifestation of HSV-2 infection in HIV+ patients

Ten of 11 HIV+ patients with gG-2-specific antibodies demonstrated by DAS-ELISA and immunoblot test contracted HIV by sexual contact. Significantly, 5 of these patients (Nos. 16, 17, 19, 37 and 56) have had recurrent symptomatic outbreaks of genital or anogenital herpes. Another 5 patients were asymptomatic and probably unaware of their HSV-2 infection. For 1 patient the history of genital herpes was unknown. We did not find any good correlation between the DAS-ELISA titre and clinical symptoms. For example patients Nos. 16, 17 and 19 had DAS-ELISA titres of gG-2 antibodies equal to 6,400 and all of them had recurrent symptomatic outbreaks. On the other hand, patient No. 5 had DAS-ELISA titre of gG-2 antibodies of 25,000 but had no history of symptomatic genital herpes. However, none of the patients with DAS-ELISA titre of gG-2 antibodies lower or equal to 3,200 had symptomatic HSV-2 outbreaks (Table 1).

For all HIV– individuals including those with gG-2-specific antibodies, the medical history of genital herpes was not available.

Discussion

The presented results showed that HSV-2 infection was more common in HIV+ than in HIV– individuals. HSV-2 infection was demonstrated by testing human sera for the presence of antibodies to gG-2. For this purpose we developed accurate gG-2-specific DAS-ELISA and immunoblot test based on our HSV-specific MoAbs described earlier (Bystrická *et al.*, 1991). A similar strategy in detecting antibodies to gG-2 has been used by Safrin *et al.* (1992), Ashley *et al.* (1988) and others. In general, there was a satisfactory correlation between the DAS-ELISA and immunoblot test results in our experiments. However, in the extremely sensitive DAS-ELISA, titres of gG-2-specific antibodies were probably overestimated and very low titres could be misinterpreted. Ashley *et al.* (1988) and Bergstrom and Trybala (1996) have found the immunoblot test very sensitive and reliable for detection and typing of antibodies to HSV-1, 2 in human sera. Our results bring further support for the notion that the immunoblot test is accurate and more reliable than the DAS-ELISA for serosurveys and diagnosis of HSV-2 infection.

High incidence of gG-2-specific serum antibodies in the group of HIV+ patients described here confirms and extends previous reports that the HSV-2 infection is an important risk factor for HIV transmission (Holmberg *et al.*, 1988; Hook *et al.*, 1992; Safrin *et al.*, 1992; Dada *et al.*, 1998). Schacker *et al.* (1998) have recently showed that HIV-1 virions could be consistently detected in genital sores caused by HSV-2; this most important finding strongly suggests that genital herpes infection apparently increases the efficiency of the sexual transmission of HIV-1. According to our knowledge, there is only one controversial study by Kingsley *et al.* (1990), in which no association between the presence of HSV-2 antibodies and HIV seroconversion among homosexual men in USA has been reported.

Ten of 11 HIV+ patients with gG-2-specific antibodies demonstrated by DAS-ELISA and immunoblot test contracted HIV by sexual contact. Five of these patients (Nos. 16, 17, 19, 37 and 56) have had recurrent symptomatic outbreaks of genital or anogenital herpes. Another 5 patients were asymptomatic, nevertheless, these patients were very likely to shed HSV-2.

Identification of patients with symptomatic as well as asymptomatic HSV-2 infection and their treatment should be required in order to prevent the spread of HSV-2 and at the same time to reduce the danger of HIV transmission, particularly in the group of HIV- people with risk behaviour, e.g. homosexual men. A therapy with anti-HSV-2 drugs has been shown to reduce outbreaks of the disease and viral shedding as well (Wald *et al.*, 1996; Sacks *et al.*, 1996). Finally, new preventive measures such as the use of recombinant glycoprotein vaccine for HSV-2 can provide a long-term protection against infection with HSV-2 (Straus *et al.*, 1994; Langenberg *et al.*, 1995).

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References

Ashley RL, Militoni J, Lee F, Nahmias A, Corey L (1988): Comparison of Western blot (Immunoblot) and glycoprotein G-specific immunodot enzyme assay for detecting antibodies to Herpes simplex virus types 1 and 2 in human sera. *J Clin. Microbiol.* **26**, 662–667.

Bergstrom T, Trybala E (1996): Antigenic differences between HSV-1 and HSV-2 glycoproteins and their importance for type-specific serology. *Intervirology* **39**, 176–184.

Bystrická M, Vančíková M, Kasalová M, Rajčáni J, Košťál M, Murányiová M, Poláková K, Russ G (1991): Type-common and type-specific monoclonal antibodies to herpes simplex virus types 1 and 2. *Acta Virol.* **35**, 152–164.

Dada AJ, Diamondstone L, Quinn TC, Blattner WA, Biggar RJ (1998): A serosurvey of Haemophilus ducreyi, syphilis, and Herpes simplex virus type 2 and their association with human immunodeficiency virus among female sex workers in Lagos, Nigeria. *Sex. Trans. Dis.* **25**, 237–242.

Eberle R, Mou SW (1983): Relative titers of antibodies to individual polypeptide antigens of Herpes simplex virus type 1 in human sera. *J. Infect. Dis.* **148**, 436–444.

Fleming DT, McQuillan GM, Johnson RE, Nahmias AJ, Aral SO, Lee FK, St. Louis ME (1997): Herpes simplex virus type 2 in the United States, 1976 to 1994. *N. Engl. J. Med.* **337**, 1105–1111.

Forsgren M, Skoog E, Jeansson S, Olofsson S, Giesecke J (1994): Prevalence of antibodies to Herpes simplex virus in pregnant women in Stockholm in 1969, 1983 and 1989: implications for STD epidemiology. *Int J STD & AIDS* **5**, 113–116.

Griffiths PD (1998): Studies of viral co-factors for human immunodeficiency virus *in vitro* and *in vivo*. *J. Gen. Virol.* **79**, 213–220.

Hashido M, Lee FK, Inouye S, Kawana T (1997): Detection of Herpes simplex virus type-specific antibodies by an enzyme-linked immunosorbent assay based on glycoprotein G. *J. Med. Virol.* **53**, 319–323.

Halbert SP, Kiefer DJ, Friedman-Kien AE, Poiesz B (1986): Antibody levels for Cytomegalovirus, Herpes simplex virus, and Rubella in patients with acquired immune deficiency syndrome. *Clin. Microbiol.* **23**, 318–321.

Ho DWT, Field PR, Sjogren-Jansson E, Jeansson S, Cunnigham AL (1992): Indirect ELISA for the detection of HSV-2 specific IgG and IgM antibodies with glycoprotein G (gG-2). *J. Virol. Methods* **36**, 249–264.

Holmberg SD, Stewart JA, Gerber AR, Byers RH, Lee FK, O'Malley PM, Nahmias AJ (1988): Prior Herpes simplex virus type 2 infection as a risk factor for HIV infection. *JAMA* **259**, 1048–1050.

Hook EW, Cannon RO, Nahmias AJ (1992): Herpes simplex virus infection as a risk factor for Human immunodeficiency virus infection in heterosexuals. *J. Infect. Dis.* **165**, 251–255.

Kingsley LA, Armstrong J, Rahman A, Ho M, Rinaldo CR (1990): No association between Herpes simplex virus type-2 seropositivity or anogenital lesions and HIV seroconversion among homosexual men. *J. Acq Immune Def. Syn.* **3**, 773–779.

Koutsky LA, Ashley RL, Holmes KK, Stevens CE, Critchlow CW, Kiviat N, Lipinski CM, Wolner-Hanssen P, Corey L (1990): The frequency of unrecognized type 2 Herpes simplex virus infection among women. *Sex. Trans. Dis.* **17**, 90–94.

Langenberg AGM, Burke RL, Adair SF, Sekulovich R, Tigges M, Dekker CL, Corey L (1995): A recombinant glycoprotein vaccine for Herpes simplex type 2 : Safety and efficacy. *Ann Intern. Med.* **122**, 889–898.

Langenberg A, Benedetti J, Jenkins J, Ashley R, Winter C, Corey L (1989): Development of clinically recognizable genital lesions among women previously identified as having "asymptomatic" Herpes simplex type 2 infection. *Ann. Intern. Med.* **110**, 882–887.

Liljequist JA, Trybala E, Svennerholm B, Jeansson S, Sjogren-Jansson, Bergstrom T (1998): Localization of type-specific epitopes of Herpes simplex virus type 2 glycoprotein G recognized by human and mouse antibodies. *J. Gen. Virol.* **79**, 1215–1224.

Safrin S, Arvin A, Mills J, Ashley R (1992): Comparison of the Western immunoblot assay and a glycoprotein G enzyme immunoassay for detection of serum antibodies to Herpes simplex virus type 2 in patients with AIDS. *J. Clin. Microbiol.* **30**, 1312–1314.

Sacks SL, Aoki FY, Diaz-Mitoma F, Sellors J, Shafran SD (1996): Patient-initiated, twice-daily oral famciclovir for early recurrent genital herpes: a randomized, double-blind multicenter trial. *JAMA* **276**, 44–49.

Schacker T, Ryncarz AJ, Goddard J, Diem K, Shaughnessy M, Corey L (1998): Frequent recovery of HIV-1 from genital herpes simplex virus lesions in HIV-1-infected men. *Dermatology* **194**, 93–97.

Straus SE, Corey L, Burke RL, Savarese B, Barnum G, Krause PR, Kost RG Meier JL, Sekulovich R, Adair SF, Dekker CL (1994): Placebo-controlled trial of vaccination with recombinant glycoprotein D of Herpes simplex virus type 2 for immunotherapy of genital herpes. *Lancet* **343**, 1460–1463.

Wald A, Zeh J, Barnum G, Davis LG, Corey L (1996): Suppression of subclinical shedding of Herpes simplex virus type 2 with acyclovir. *Ann. Intern. Med.* **124**, 8–15.

Wasserheit JN (1992): Epidemiological synergy: interrelationhips between human immunodeficiency virus infection and other sexually transmitted diseases. *Sex. Trans. Dis.* **19**, 61–77.