PREVALENCE OF ANTIBODIES TO HERPES SIMPLEX VIRUS 2 AMONG HOMOSEXUAL MEN EITHER POSITIVE OR NEGATIVE FOR HUMAN IMMUNODEFICIENCY VIRUSES IN SLOVAKIA

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Summary. – We determined the prevalence of antibodies to herpes simplex virus 2 (HSV-2, HSV-2 antibodies) in sera of homosexual men either positive for human immunodeficiency virus 1 (HIV-1, HIV^+, a group of 27 sera) or negative for HIV-1 and HIV-2 (HIV^-, a group of 52 sera) in Slovakia. Antibodies to HSV-2 glycoprotein G-2 (gG-2, gG-2 antibodies) were determined by a double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and immunoblot analysis. We found that 40% of HIV^+ and 23% of HIV^- homosexual men were positive for the gG-2 antibodies, what is 3.6 and 2.1 times higher incidence, respectively, than that in the control heterosexual population (Bystrická *et al.*, *Acta Virol.* **42**, 319–324, 1998). Identification of individuals infected with genital herpes among HIV^+ and HIV^- homosexual men should be succeeded by antiviral therapy in order to prevent transmission of HSV-2 and HIV as well in this community.

Key words: HSV-2; HSV-2 antibodies; HIV; glycoprotein G-2; immunoblot analysis; ELISA; homosexual men

Introduction

HSV-2 infection is one of the most frequently diagnosed sexually transmitted diseases. It is known that HSV-2 infection results in a seroconversion and lifelong persistence of virus-specific antibodies that may be used as an objective marker to establish the presence of genital herpes infection. An HSV-2-specific serological test has been often used to assess the prevalence of HSV-2 antibodies in population and to identify asymptomatic or subclinically infected individuals (Fleming *et al.*, 1997; Bergstrom and Trybala 1996; Koutsky *et al.*, 1990; Ashley *et al.*, 1988; Sullender *et al.*, 1988; Lee *et al.*, 1985).

Generally, sexually transmitted diseases that cause genital ulcers and inflammation such as HSV-2 infection, could contribute to transmission of HIV through the reduced epithelial barrier and infiltration of $CD4^+$ lymphocytes in herpetic lesions that are possible targets for HIV attachment and entry. On the other hand, more severe HSV-2 outbreaks and more frequent viral shedding are common in persons infected with HIV. Thus, it appears that HSV-2 and HIV infections have a bidirectional interaction in which one exacerbates or predisposes the other (Severson and Tyring, 1999; Griffiths 1998; Schacker *et al.*, 1998; Wasserheit *et al.*, 1992; Hook *et al.*, 1992). In many studies, the presence

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Abbreviations: AIDS = acquired immunodeficiency syndrome; DAS-ELISA = double-antibody sandwich enzyme-linked immunosorbent assay; gB = glycoprotein B; gB-2 = HSV-2 gB; gB-1,2 = HSV-1/HSV-2 gB; gG = glycoprotein G; gG-2 = HSV-2 gG; HIV-1 = human immunodeficiency virus 1; HIV-2 = human immunodeficiency virus 2; HIV^+ = HIV-1 positive, HIV-2 negative; HIV^- = HIV-1/2-negative; HSV-2 = herpes simplex virus 2; MAb = monoclonal antibody; PBS = phosphate-buffered saline; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate

of HSV-2 infection has been shown to be associated with a higher risk of acquiring HIV infection. Indeed, the rate of HSV-2 infection appears particularly high among homosexual and bisexual men with HIV infection (Hook *et al.*, 1992; Stamm *et al.*, 1988; Holmberg *et al.*, 1988).

The objective of the present study was to determine the occurrence of HSV-2 antibodies in groups of 27 HIV$^+$ and 52 HIV$^-$ homosexual men and to compare the incidence of HSV-2 antibodies in these groups with that in normal heterosexual population in Slovakia. The greatest value of such a serological testing may reside in identification of HSV-2-infected individuals succeeded by antiviral therapy and counseling in order to prevent transmission of HSV-2 and HIV among men with homosexual orientation.

Materials and Methods

Cells, viruses, monoclonal antibodies (MAbs) and antigen extracts. Vero cells were propagated and maintained as described previously (Bystrická *et al.*, 1991). HSV-2 strain Praha was acquired from SEVAC, Czech Republic. MAb 303 against HSV-2 gG (gG-2), MAb 499 against HSV-2 gB (gB-2), and MAbs T111 and 170 against both HSV-1 and HSV-2 gB (gB-1,2) were prepared and characterized as described elsewhere (Bystrická *et al.*, 1991). Preparation of extracts of infected or non-infected cells by use of a detergent was described previously (Bystrická *et al.*, 1998).

Human sera. Twenty-seven sera of HIV$^+$ and 52 sera of HIV$^-$ homosexual men were collected at the National Reference Center for Prevention of AIDS/HIV in Bratislava, Slovakia. Each serum was tested twice by standard ELISA (Abbott Laboratories) for the presence of antibodies to HIV-1 and HIV-2. Positive ELISA results were confirmed by immunoblot analysis (HIV Western Blot, Murex). The HIV$^+$ sera were positive for the HIV-1 antigen only, while the HIV$^-$ sera were negative both to HIV-1 and HIV-2 antigens.

DAS-ELISA was employed for assay of gG-2 and gB-1,2 antibodies (Bystrická *et al.*, 1998). A checkerboard titration was performed to determine the optimal dilutions of ascitic fluids, cell extracts and the anti-human conjugate. One positive and one negative reference human serum were used throughout the study as markers of presence or absence of HSV-2 antibodies. The titer of the antibodies of a serum was defined as the reciprocal value of the highest serum dilution at which the absorbance value was at least 2 times higher than that of the reference HSV-2-negative serum at the same dilution.

Purification of gG-2 was done by immunoaffinity chromatography as described previously (Bystrická *et al.*, 1998). Briefly, an affinity column was made with 5 mg of purified MAb 303 and 1 g of CNBr-Sepharose (Pharmacia) according to the manufacturer´s instructions. About 350 million cells were extracted for 30 mins on ice and centrifuged. The supernatant was added to the CNBr-Sepharose coupled with MAb 303 and incubated overnight at 4°C under constant shaking. 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 and dialyzed against phosphate-buffered saline (PBS) for 3 days.

Immunoblot analysis. All procedures used in immunoblot analysis except the incubation with the second antibody and detection were described elsewhere (Bystrická *et al.*, 1991). Briefly, 1 ml of purified gG-2 in PBS with approximate protein concentration of 0.05 mg/ml was subjected to a standard polyacrylamide gel (8.5%) electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Then the gel was incubated in a transfer buffer (25 mmol/l Tris pH 8.3, 192 mmol/l glycine and 10% methanol) for 1 hr and blotted onto a nitrocellulose membrane (Schleicher and Schuell, 0.22 μm). After blotting, the remaining binding sites were blocked by incubating the blot in PBS containing 5% nonfat dry milk for 2 hrs. Blot strips were incubated with human sera diluted 1:100 or 1:200 in PBS containing 2% nonfat dry milk for 2 hrs with constant agitation. Positive and negative reference sera diluted 1:200 and 1:100, respectively, and MAb 303 (as ascitic fluid) diluted 1:2000 were used as controls. The strips were washed 4 x 10 mins in PBS under constant agitation. Detection of the bound antibodies was accomplished by incubation of the strips with an anti-human (Dako, Denmark) or anti-mouse (Sevac, Czech Republic) conjugate diluted 1:4,000 and 1:2,000, respectively, in PBS containing 2% nonfat dry milk and 0.1% Nonidet P-40 for 1 hr at room temperature followed by washing 5 x 10 mins in PBS containing 0.1% Nonidet P-40. Visualization of the bound antibodies was performed by exposition of the strips treated for 1 min with a chemiluminiscence reagent (Amersham, Life Sciences) to a X-ray film (Fuji, Super RX) for different periods of time.

As usually done with scarce antigens, the strips probed with negative sera were erased by incubation in a stripping buffer (62.5 mmol/l Tris-HCl pH 6.7, 2% SDS, and 100 mmol/l 2-mercaptoethanol) at 70°C for 30 mins with occasional agitation (Kaufmann *et al.*, 1987) and reprobed with another human serum and an anti-human conjugate with the same sensitivity.

Results

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

For assaying gG-2 and gB-1,2 antibodies a DAS-ELISA with corresponding MAbs captured on a plate was used. Of 27 HIV$^+$ homosexual men tested, 14 individuals showed high levels of gG-2 antibodies ranging in titers from 6,400 to 100,000. The remaining patients showed low (1,600 to 3,200) or negative titers (Table 1). Thirty of 52 HIV$^-$ homosexual men showed high titers of gG-2 antibodies ranging from 6,400 to 100, 000. The rest of the patients in this group showed lower or negative titers (Table 2). All the sera with high titers of gG-2 antibodies showed also high titers of gB-1,2 antibodies (6,400 to 200,000) confirming the fact that the patients were exposed to HSV-1 and/or HSV-2 and developed strong immune response against these viruses (Eberle and Mou, 1983).

Table 1. Reactivity of sera of HIV⁺ homosexuals in DAS-ELISA and immunoblot analysis

No. of patients in the group	Titer of gG-2 Antibodies*	Titer of gB-1,2 Antibodies*	Immunoblot assay for gG-2
11	6,400–100,000	6,400–200,000	Positive
3	6,400–100,000	6,400–200,000	Negative
13	<1,600–3,200	<1,600–25,000	Negative
Positive reference serum	100,000	200,000	Positive
Negative reference serum	<1,600	<1,600	Negative

*DAS-ELISA.

Table 2. Reactivity of sera of HIV⁻ homosexuals in DAS-ELISA and immunoblot analysis

No. of patients in the group	Titer of gG-2 Antibodies*	Titer of gB-1,2 Antibodies*	Immunoblot assay for gG-2
12	6,400–100,000	6,400–200,000	Positive
18	6,400–100,000	6,400–200,000	Negative
22	< 1,600–3,200	<1,600–50,000	Negative
Positive reference serum	100,000	200,000	Positive
Negative reference serum	<1,600	<1,600	Negative

*DAS-ELISA.

Immunoblot assay of gG-2 antibodies

To confirm the presence or absence of gG-2 antibodies in the sera, their reactivity against gG-2 in DAS-ELISA was tested also by immunoblot analysis.

First, blot strips with purified and electrophoretically separated gG-2 were allowed to react with MAb 303 (dilution 1:2000) raised against this glycoprotein. After incubation with an anti-mouse conjugate diluted 1:2,000 we detected presence of a band with apparent M_r of 115 K, which represents the mature O-glycosylated gG-2 (Balachandran and Hutt-Fletcher, 1985). In addition, we detected a group of bands of 63–70 K that may represent cleavage intermediates in normal processing of gG-2. (Fig. 1, lane I). Detection of the 115 K band (mature gG-2) was the criterion for identification and confirmation of gG-2 antibodies in tested sera.

Next, groups of 27 HIV⁺ and 52 HIV⁻ sera were tested in order to confirm the presence of gG-2 antibodies detected previously by DAS-ELISA. The blot strips with gG-2 were allowed to react with sera diluted 1:100 or 1:200 and then with an anti-human conjugate diluted 1: 4,000. Fig. 1 shows the representative results obtained with 5 positive sera (lanes A–E) reacting with the 115 K band, and 3 negative sera showing a non-specific reaction with a group of bands of 50–55 K (lanes F–H). Positive (lane J) and negative (lane K) reference human sera were also included. It is evident that the positive sera (lanes A–E) and the positive reference human serum (lane J) reacted with the same 115 K band as the mouse MAb 303 (lane I). The negative reference human serum failed to show any reactivity with purified gG-2 (lane K).

In the group of HIV⁺ patients most of the sera with high DAS-ELISA titers of gG-2 antibodies were also positive in immunoblot analysis. We detected 11 of 27 (about 40%) sera as positive with both methods (Table 1). However, in the group of 52 HIV⁻ sera, the concordance between high levels of antibodies detected by DAS-ELISA and positivity in immunoblot analysis was poor. Out of 30 sera with high DAS-ELISA titers we detected only 12 reacting with the 115 K band in immunoblot analysis (Table 2).

Most of the remaining 18 sera with high DAS-ELISA titers of gG-2 antibodies reacted non-specifically with groups of distinct bands of about 50–55 K, while there was no reactivity with the 115 K mature gG-2 (Fig. 1, lanes F–H). According to our criteria these sera cannot be considered positive for the presence of gG-2 antibodies, because the reactivity to the fully glycosylated gG-2 was absent.

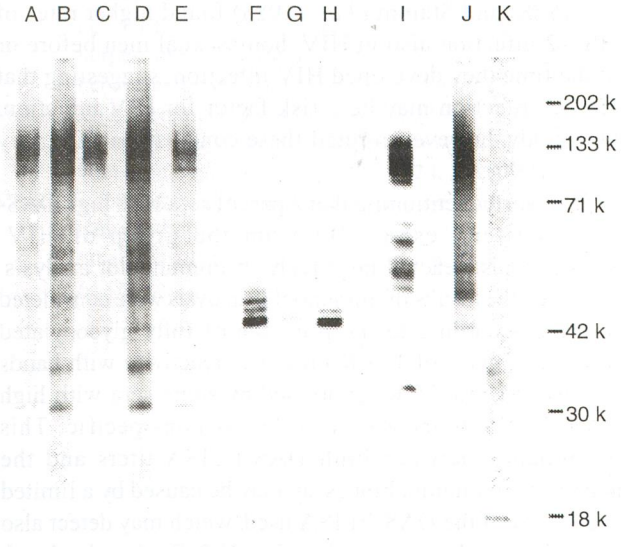

Fig. 1
Immunoblot analysis of human sera
Purified gG-2 was subjected to SDS-PAGE, blotted onto a nitrocellulose membrane and probed with MAb 303 and the tested human sera. The M_r values of size markers are indicated on the right. The tested human sera (diluted 1:100) positive for gG-2 antibodies (lanes A–E); the tested human sera (diluted 1:100) negative for gG-2 antibodies (lanes F–H); MAb 303 as ascitic fluid diluted 1:2,000 (lane I); positive reference human serum diluted 1:200 (lane J); negative reference human serum diluted 1:100 (lane K).

These results also confirmed our view that even high DAS-ELISA titers can be misleading and may represent a non-specific reaction and therefore the presence of gG-2 antibodies must be reconfirmed by immunoblot analysis.

Discussion

The present study was designed to determine the seroprevalence of genital herpes antibodies among groups of HIV^+ and HIV^- homosexual men in Slovakia. In our experiments the presence of gG-2 antibodies detected in DAS-ELISA was tested by a reference technique – the immunoblot analysis using purified gG-2 as an antigen.

We found 40% of tested HIV^+ homosexual men positive for gG-2 antibodies by DAS-ELISA and immunoblot analysis as well. In the reference group of HIV^- homosexual men, gG-2 antibodies were detected only in 23% of individuals by both immunoassays. The prevalence of HSV-2 antibodies in normal healthy heterosexual population in Slovakia is 11% (Bystrická *et al.*, 1998) that means that the rates of HSV-2-infected patients among HIV^+ and HIV^- homosexual men is 3.6 and 2.1 times higher, respectively. Similarly to our results, significantly higher incidence of HSV-2 antibodies in sera of HIV^+ patients has been demonstrated also by others (Dobbins *et al.*, 1999; Hook *et al.*, 1992; Safrin *et al.*, 1992). In addition, Holmberg *et al.* (1988) and Stamm *et al.* (1988) found higher rates of HSV-2 infection also in HIV^- homosexual men before or at the time they developed HIV infection, suggesting that HSV-2 infection may be a risk factor for HIV infection. One study, however, refuted these conclusions (Kingsley *et al.*, 1990).

It is worth mentioning that a part of sera with high DAS-ELISA titers, especially from the group of HIV^- homosexuals, reacted negatively in immunoblot analysis. However, the results of immunoblot analysis were considered positive according to the presence of fully glycosylated product of gG-2 of 115 K only. The reactivity with bands of lower M_r (50–55 K) expressed by some sera with high DAS-ELISA titers was regarded as non-specific. This discrepancy between high DAS-ELISA titers and the negativity in immunoblot assay may be caused by a limited specificity of the DAS-ELISA used, which may detect also antibodies to other antigens besides gG-2. On the other hand, there is also a good chance of obtaining false-positive results especially in non-HSV-2-infected patients with high titers of HSV-1 antibodies, when a certain degree of "spill-over" reactivity can be present in gG-2 assays (Bergstrom and Trybala, 1996). Other workers (Ho *et al.*, 1993; Safrin *et al.*, 1992; Ashley *et al.*, 1988) reported also much greater sensitivity and reliability of immunoblot analysis as compared with indirect ELISA.

A key factor in the spread of genital herpes infections is a high proportion of undiagnosed asymptomatic infections (Ashley and Wald, 1999; van de Laar *et al.*, 1998; Koutsky *et al.*, 1990). In our view, reliable identification of HSV-2-infected individuals requires immunoblot assay of antibodies, because a reported history of genital herpes or positive ELISA results are poor predictors of HSV-2 infection. Identified persons with genital herpes infection (especially among HIV^- homosexuals) should be targeted for treatment and prevention of HSV-2 transmission. The acyclovir therapy has been demonstrated to reduce the time for healing and virus shedding from patients with initial and recurrent genital herpes (Wald *et al.*, 1996; Kaplowitz *et al.*,1991). Treatment of genital herpes and other sexually transmitted diseases at the same time actually decreases the rates of HIV infection (Severson and Tyring, 1999).

Finally, counseling of HIV^+ and HIV^- homosexual men regarding possible risk of HIV acquisition during recurrent genital herpes, abstinence during recurrences or treatment with acyclovir may contribute to the control of HIV transmission in Slovakia.

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References

Ashley RL, Wald A (1999): Genital herpes: Review of the epidemic and potential use of type-specific serology. *Clin. Microbiol. Rev.* **12**, 1–8.

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.

Balachandran N, Hutt-Fletcher M (1985): Synthesis and processing of glycoprotein gG of herpes simplex virus type 2. *J. Virol.* **54**, 825–832.

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, Soláriková L, Gašparovičová L, Staneková D, Mokráš M, Kováčová E, Sabó A, Russ G (1998): Antibody responses to the herpes simplex virus type 2 glycoprotein G in sera of human immunodeficiency virus-infected patients in Slovakia. *Acta Virol.* **42,** 319–324.

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

Dobbins JG, Mastro TD, Nopkesorn T, Sangkharomya S, Limpakarnjanarat K, Weniger BG, Schmid DS (1999):

Herpes in the time of AIDS: A comparison of the epidemiology of HIV-1 and HSV-2 in young men in nothern Thailand. *Sex. Trans. Dis.* **26**, 67–74.

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.

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

Ho DWT, Field PR, Irving WL, Packham DR, Cunningham AL (1993): Detection of immunoglobulin M antibodies to glycoprotein G-2 by Western blot (immunoblot) for diagnosis of initial herpes simplex virus type 2 genital infections. *J. Clin. Microbiol.* **31**, 3157–3164.

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.

Kaplowitz LG, Baker D, Gelb L (1991): Prolonged continuous acyclovir treatment of normal adults with frequently recurring genital herpes simplex virus infection. *JAMA* **265**, 747–751.

Kaufmann SH, Ewing CM, Shaper JH (1987): The erasable western blot. *Anal. Biochem.* **161**, 89–95.

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.

Lee FK, Coleman RM, Pereira L, Bailey PD, Tatsuno M, Nahmias AJ (1985): Detection of Herpes simplex virus type 2-specific antibody with glycoprotein G. *Clin. Microbiol.* **22**, 641–644.

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.

Severson JL, Tyring SK (1999): Relation between herpes simplex viruses and human immunodeficiency virus infections. *Arch. Dermatol.* **135**, 1393–1397.

Schacker T, Ryncarz AJ, Goddard J, Diem K, Shaghnessy M, Corey L (1998): Frequent recovery of HIV-1 from genital herpes simplex virus lesions in HIV-infected men. *JAMA* **280**, 61–66.

Sullender WM, Yasukawa LL, Schwartz M, Pereira L, Hensleigh PA, Prober CG, Arvin AM (1988): Type-specific antibodies to Herpes simplex virus type 2 (HSV-2) glycoprotein G in pregnant women, infants exposed to maternal HSV-2 infection at delivery, and infants with neonatal herpes. *J. Inf. Dis.* **157**, 164–171.

Stamm WE, Handsfield H, Rompalo AM *et al.* (1988): The association between genital ulcer disease and acquisition of HIV infection in homosexual men. *JAMA* **260**, 1429–1433.

van de Laar MJW, Termorshuizen F, Slomka MJ, van Doornum GJJ, Ossewaarde JM, Brown DWG, Coutinho RA, van den Hoek JAR (1998): Prevalence and correlates of herpes simplex virus type 2 infection: evaluation of behavioural risk factors. *Int. J. Epidemiol.* **27**, 127–134.

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. Transm. Dis.* **19**, 61–77.