Symptomatic Reactivation of HSV Infection Correlates with Decreased Serum Levels of TNF-α

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ABSTRACT

Background: Herpes simplex viruses (HSV) are human pathogens that establish lytic and latent infections. Reactivation from latency occurs intermittently, which represents a life-long source for recurrent infection. The role of immune factors in the control of recurrent symptomatic HSV lesions is complex and the exact role of cytokines remains unclear. **Objective:** To assess the levels of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) along with anti-herpetic IgG and IgM, in the symptomatic reactivation of HSV infection. **Methods:** Thirty-six patients with recurrent symptomatic herpes infection were selected as the study group and thirty-two healthy individuals with no history of symptomatic labial herpes infection enrolled as the control group. Skin swabs were obtained from lip and skin lesions for viral culture. Confirmation of HSV cytopathic effect was carried out using PCR assay. The levels of TNF-α, IL-10, IgG and IgM were measured using ELISA. **Results:** The level of TNF-α was significantly lower in individuals with recurrent symptomatic herpes infection in comparison with the controls ($p=0.04$). Also a significant elevation was observed in the levels of specific IgG in patients compared to controls ($p<0.05$). **Conclusion:** The decreased level of TNF-α and increased levels of IgG in individuals with a history of symptomatic reactivation of HSV infection is suggestive of a probable shift in favor of the Th2 immune response.

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INTRODUCTION

Herpes simplex virus (HSV) is a human pathogenic virus that causes infection through contact with mucosal membranes. Their attractions are their biologic properties, particularly their abilities to cause a variety of infections, remain latent in their host for life, and reactivate to cause lesions at or near the site of the initial infection. After primary HSV infection of epithelial cells, the virus establishes latency in the trigeminal ganglia (1).

HSV-1 establishes latent infections within the peripheral neurons, although a vigorous immune response during primary infection is elicited. Viral gene expression and its replication leading to infectious progeny virus production are induced by either physical or psychological stress, and infections (2,3). Reactivation is sometimes recognized by a recurrent symptomatic disease and/or virus transmission (4).

In spite of the clinical and experimental studies on the host defense responses against HSV (5,6), neither single host factor nor a set of host factors capable of preventing virus symptomatic reactivation have been identified (4). Host defense response against HSV-1 reactivation is affected by not only viral factors but also host factors such as cytokines as the regulator of the immune response (7).

Tumor necrosis factor-α (TNF-α), a pleiotropic factor critical for the development of inflammation, is synthesized mainly by macrophages in response to various stimuli like viral infections (8,9). TNF-α may possibly influence the course of HSV-1 infection since it has been shown to demonstrate antiviral activities in several cell lines infected with animal viruses, and it may do the same with HSV-1 infected mice (10-13). TNF-α and interferon-γ (IFN-γ) play protective roles in acute HSV-1 infected mice (5,7).

In contrast to the pro-inflammatory characteristics of TNFs, other cytokines such as interleukin-10 (IL-10) are known to have anti-inflammatory properties. IL-10 has been shown to relieve HSV induced inflammation (14,15). IL-10 is an immunosuppressive cytokine and is necessary for the protection against pathogenic hyper-inflammatory responses in the CNS in HSV-1 infection (16).

The exact roles of TNF-α and IL-10 in HSV-1 recurrence in human beings are still unclear. The present study was conducted to investigate the relationship between symptomatic reactivation of HSV infection and the serum levels of TNF-α and IL-10 as the two important cytokines affecting the inflammatory process in viral infection. Also, anti-HSV IgG and IgM were detected in the symptomatic subjects along with the asymptomatic controls.

MATERIALS AND METHODS

Subjects. The specimens were collected from the patients admitted to Departments of Dermatology, Faghihi Hospital, Shiraz, Iran, from September 2008 to July 2010. Thirty-six patients (10 male , 26 female) with recurrent symptomatic herpes infection and a mean age of 35.8 ± 13.1 years were enrolled as the study group, while 32 healthy individuals (9 male, 23 female) with no prior history of recurrent symptomatic labial herpes infection and a mean age of 36.2 ± 8.1 years were enrolled as the control group. The control group was matched based on age and sex. The study was approved by the Ethics Committee of Shiraz University of Medical Sciences and written informed consent was obtained from each participant.

Specimen Collection and Isolation of HSV. Skin swabs were obtained from the lips and skin lesions for viral culture and DNA extraction. The swabs were immersed in phosphate buffered saline (PBS) and transferred to the laboratory, promptly, in cold containers. Vero cell line (Razi Institute, Hesarak-Iran) were prepared in Dulbecco's Modified Eagle Medium, DMEM, (Sigma, Germany) supplemented with 4% fetal calf serum. Cells were cultured in a humidified incubator at 37°C containing 5% CO_2. Skin swabs were inoculated in Vero cells, incubated at 37°C and monitored for cytopathic effects (CPE) 48 h later. Characteristic CPE of HSV cell infection included ballooning of infected cells and formation of multinucleated giant cells.

Polymerase Chain Reaction. DNA of infected cultured cells which showed characteristic HSV CPE was extracted using a genomic DNA extraction kit (Bioneer, Korea) and kept at -25°C till performing the PCR. General primers (Table 1) for HSV (Takapouzist, Iran) were used to amplify a highly conserved regions within the DNA polymerase gene (17).

Table 1. Primers used to amplify DNA polymerase gene of HSV-1.

Primer Name	Primer Sequence	PCR Product Size (bp)
HSV-GF	5´-GTGTTCGACTTTGCCAGCCTCTAC-3	223
HSV-1-2R	5´-GACTGGCTCGCCATGCGAAAGC-3´	

Positive control of HSV-1 for PCR was prepared from the primary clinical isolate confirmed by neutralization test using guinea pig anti-HSV-1 serum (NIH, USA) and monoclonal (D and G) anti HSV-1 antibodies. PCR mixture without DNA, containing distilled water instead, served as negative control. PCRs for confirmation of HSV were performed in a thermocycler (Eppendorf, Germany) in a reaction volume of 50 µl containing 5 µl 10x PCR buffer, 1.5 µl of 50 mM $MgCl_2$, 0.2 µM of each dNTPs, 1 U Taq DNA Polymerase (CinnaGen, Iran), 10 pmol/µl of each primer of and 5 µl of template DNA. After initial denaturation at 95°C for 5 min, PCR products were amplified by 35 cycles of denaturation at 94°C for 2 min, annealing at 60°C for 45 s and extension at 72°C for 45 s, with a final extension step at 72°C for 10 min. The amplified products were detected by staining with ethidium bromide (CinnaGen, Iran) in 2% agarose gel (Fermentas, Kyiv, Ukraine) and photographed under UV light (18).

Blood Samples. Blood samples were taken into tubes without anticoagulant and allowed to clot for 2 h. Sera were separated following centrifugation at 3000 rpm for 10 min and stored at -20°C until used.

Immunoglobulin and Cytokine Assay. IgG and IgM anti-HSV antibodies were detected in all serum samples using a commercial ELISA kit (IBL, Hamburg, Germany). The serum levels of IL-10 and TNF-α were determined in duplicate using a commercial ELISA kits (BenderMed, Vienna, Austria). The minimum detectable levels of IL-10 and TNF-α were 0.66 and 4.3 pg/mL, respectively.

Statistical Analysis. Data were analyzed by Mann–Whitney U, Kruskal-Wallis and Chi-square test using the SPSS software. P value less than 0.05 was considered as statistically significant.

RESULTS

All the skin/lip samples in the symptomatic group were positive for HSV, confirmed by the appearance of CPE in cell culture and PCR. All samples, as well as the positive controls, produced a specific 223 bp band in PCR.

Table 2. Mean ± SD of the serum IgG, IgM, TNF-α and IL-10 levels in diseased and control groups.

Parameter	Patients	Controls	P-Value
IgG (U/mL)	94.75 ± 32.18^{a}	55.01 ± 44.66^{b}	0.0001
IgM (U/mL)	6.20 ± 3.50^{a}	5.72 ± 4.38^{a}	0.2
IL-10 (pg/mL)	1.26 ± 1^{a}	1.25 ± 0.41^{a}	0.97
TNF-α (Pg/mL)	6.61 ± 8.08^{a}	11.59 ± 13.73^{b}	0.04

a and b: Different letters in each row reveals a significant difference.

Negative control produced no DNA product following PCR amplification. Mean ± SD of serum IgG, IgM, TNF-α and IL-10 levels are presented in Table 2. The levels of IgG and TNF-α were significantly different between the patients and controls ($p<0.05$). However, no significant difference was observed in IL-10 and IgM titer between the two groups.

Table 3. The Relationship between the age and recurrence rate, per year, in the studied patients.

Demographic Data		No. of Patients with 1 or ≥2 of Recurrence Rate/Year		P-Value
		1	≥2	
Mean Age	20.23	24	0	0.21
	12.29	0	7	
	18.90	0	5	
Gender	Female	16	10	0.438
	Male	8	2	

Anti-HSV IgG was detected in all participants from both groups. The serum level of IgG was significantly higher in the patients compared with controls ($p<0.0001$). Anti-HSV IgM was detected in 4 (11%) of the patients and 3 (9.3%) of the controls. There

was no significant difference in the serum level of IgM between the two groups (p=0.2). The level of TNF-α was significantly lower in the diseased group in comparison with the controls (p<0.04). No significant difference was observed in IL-10 level in patients in comparison with the controls (p=0.97).

As shown in Table 3, no significant relationship was observed between age and recurrence rate (p=0.212). The recurrence rate was found to be higher in women compared to men. However, no significant relationship was found between sex and recurrence rate (p=0.438) as presented in Table 3. There was also no significant relationship between serum levels of TNF-α, IL-10, IgG, IgM and the recurrence rate (p>0.05).

DISCUSSION

Herpes simplex virus-1 is a human alpha herpes virus commonly found throughout the world (4). Transmission via sensory nerves to the spinal or trigeminal ganglia occurs after skin or mucosal infection (19). HSVs are human pathogens that establish lytic and latent infections. Reactivation from latency occurs intermittently which represents a life-long source of recurrent infection (20).

The ability of HSV to cause a persistent latent infection results in the disability of the immune system to complete clearance of infection and, in some individuals, in controlling reinfection (21). HSV starts by neutralizing the innate immunity and results in a viral infection that is characterized by a dynamic balance between HSV and the innate immune system (20). Both cellular and humoral immunity play roles in preventing latent and severe HSV infection (22-24). Shedding of HSV may be entirely asymptomatic or small/atypical lesions may be recognized (25). The mechanism of virus immune evasion in recurrent disease remains unclear (26).

Pro-inflammatory immune cells are involved in immune mechanisms creating protection against HSV-1 infections (27). Key regulators of the inflammatory and immune responses are cytokines including interleukins, interferon, colony stimulating factors, and tumor necrosis factors (28). They may be involved in the production of symptomatic herpes virus recurrence. Several reports have investigated the role of cytokines in HSV infection (4,6,27,29,30).

In our study, the presence of detectable levels of IgG in the control group reveals that although these individuals experienced virus exposure, they did not develop a symptomatic disease. A significant decrease was observed in the level of TNF-α in symptomatic patients in comparison with individuals who experienced HSV-1 exposure but did not develop symptomatic herpes labialis (p<0.04). This result indicated that TNF-α level could be affected by HSV infection and was in agreement with that of other researchers such as Minami *et al*. (2002), Minagawa *et al*. (2004), Fields *et al*. (2006), and Sergerie *et al*. (2007) who all showed that TNF-α is involved in host defense response against HSV-1 infection (4,7,29,30). However, Ohtani *et al*. (2001) reported that serum TNF- α was not affected by HSV-1 reactivated patients with facial palsy (31). These researchers would perhaps find a significant difference in TNF-α level between patients with facial palsy and the controls if a larger population was included in their study. TNF is mainly produced by activated macrophages, T lymphocytes, and natural killer cells and plays an important role in the pathological mechanisms (32). TNF has long been known to have synergistic antiviral effects together with IFNs (4).

The combination of TNF-α and IFN-γ at low levels shows strong anti-herpes effects in human corneal fibroblasts (12). TNF-α is an important part of the innate immune response to HSV-1 encephalitis and has a protective effect in HSV-1 experimental infection in mice (30,33). IFN-γ and TNF-α play important roles in acute infection and reactivation from latency (5,7,29). Following stimulation of HeLa and 86HG39 cells by IFN-γ and TNF-α, replication of HSV-2 is inhibited. In the presence of TNF-α, the antiviral effect of IFN-γ is enhanced while it was not induced by TNF-α alone (34). HSV induced inflammation in mice was decreased by TNF-α small interfering RNA (35).

We found no significant difference in the IL-10 level of HSV-infected individuals who revealed symptomatic disease compared with the controls, in whom, symptomatic disease was not developed in spite of probable reactivation. Richards *et al*. (2003) reported an increased level of IL-10 in recurrent ocular HSV infection (27). The studies of Yan *et al*. (2001), Marques *et al*. (2004) and Schneider *et al*. (2004) revealed the ability of IL-10 in relieving the inflammation induced by HSV (6,14,15). The induction of IL-10 has an important role in regulating inflammatory responses (27). IL-10 treatment decreases the incidence of HSV-1-induced blindness in mice (6,36). Administration of IL-10 antibody and disruption of the IL-10 gene were associated with an increase in the severity of HSV-1-induced corneal disease (6,15). IL-10 is considered to regulate microglial cell production of immune mediators leading to modulation of the pro-inflammatory response to HSV-1 (6). The induction of IL-10 after HSV-1 corneal infection reduces the migration of inflammatory cells to the site of infection (15,27). Nevertheless there was a low increase in the IL-10 level in our symptomatic group compared with the controls; it was not a statistically significant increase. A larger sample size would have probably led to a result similar to the ones mentioned above. It may be considered as a limitation of our study.

Understanding the role of the immune response during HSV infection is important for controlling the disease and might improve the treatment, prevent the establishment of latency, and limit virus spread after reactivation from the latent state. Regulation of immune response may be a treatment option for patients with recurrent infection.

Collectively, the decreased level of TNF-α and increased levels of IgG in individuals with a history of symptomatic reactivation of HSV infection in our study suggest that in recurrent symptomatic HSV infection, a shift in immune response occurs during which those associated with Th2 overcome that of Th1. Based on our observations, we recommend that further studies be conducted that can assess the mechanisms involved in cytokine secretion and determine related genetic polymorphism. Also, it is a requirement that such investigations be done on a larger population**.**

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