Turk J Med Sci
33 (2003) 83-87

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PCR Analysis of HSV-Negative Erythema Multiforme for the Expression of Other Herpesviruses

Received: January 17, 2003

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Abstract: *Background:* Herpes simplex virus (HSV) is the primary herpesvirus implicated to have a causal role in erythema multiforme (EM), both in cases with an antecedent herpetic infection and in idiopathic EM. However, the association of EM with several other viral infections has been clearly documented.

Objective: The aim of the present study was to investigate a hypothetical role for the remaining members of the herpesvirus family in HSV-negative cases of EM.

Methods: Fresh, unfixed lesional skin biopsies from 25 patients with HSV-negative EM were studied for the presence of Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) by using polymerase chain reaction (PCR) and nested PCR.

Results: PCR revealed the absence of specific DNA sequences for EBV, CMV, HHV-6 and HHV-7 in all lesional EM specimens analyzed.

Conclusion: These findings do not support an etiological role for non-HSV members of the herpesvirus family in HSV-negative EM.

Key Words: Erythema multiforme, Epstein-Barr virus, cytomegalovirus, human herpesvirus-6, human herpesvirus-7, polymerase chain reaction.

Introduction

Erythema multiforme (EM) is a self-limited, acute or recurrent polymorphous eruption composed of macules, papules, bullae and targetoid lesions that are symmetrically distributed but have a propensity for the distal extremities and oral mucosa (1-5). Drugs, mycoplasma and HSV infections are among the most frequently implicated causes of EM (2,3).

Infection with herpes simplex virus (HSV) is the dominant precipitating factor in the development of EM in both children and adults (1-7). Recurrent EM is preceeded by clinical lesions of recurrent HSV infection in up to 80% of patients (5,7). Both HSV-I and HSV-II have been shown to trigger EM (6). Despite this well-established clinical association, not all EM episodes are preceeded by HSV infection and not all HSV episodes are followed by EM (3,6). HSV has only rarely been cultured from EM lesions (1,5,6,8). Although polymerase chain reaction (PCR) and *in situ* hybridization (ISH) can reveal the presence of HSV DNA (1,4-8) and immunofluorescence and immunohistochemical techniques can demonstrate HSV-specific antigens in the lesional epidermis (6-8), there are cases in which no link with HSV can be demonstrated. In addition, there are documented case reports of EM in association with herpes zoster virus, Coxsackie virus, hepatitis A, B and C viruses, adenovirus, polio virus, orf, milker's nodules and HIV infection (7,9-11).

The herpesviruses are large DNA viruses with intranuclear replication (12). Their ability to directly infect lymphocytes and to establish latency in host tissues have made them the focus of investigations in dermatology (12-14).

The purpose of the present study was to elucidate a potential etiopathogenetic role for other members of the herpesvirus family in HSV-negative cases of EM.

Materials and Methods

Selection of Samples

This study involved 25 of 42 biopsy samples obtained from patients with EM diagnosed in the Dermatology Department of the Faculty of Medicine Kırıkkale

University at between July 1998 and July 2002. In a previous study performed by our department, these 25 samples were shown to be HSV-negative by PCR. In brief, the diagnosis of EM was based on typical clinical features and histology. Two punch biopsies were obtained from each patient; one was fixed in formalin, embedded in paraffin and routinely processed for histopathologic examination and the other was quick-frozen in liquid nitrogen and kept in a refrigerator at – 70 °C until PCR analysis.

DNA extraction

DNA extraction and PCR procedures were performed in the PCR laboratory of the Department of Microbiology and Clinical Microbiology of the School of Medicine at Gulhane Military Medical Academy. A standard protocol for the extraction of DNA was used (15). In brief, skin specimens were minced and pretreated by gentle mixing with 50 µl of proteinase K (100 µg/ml, Sigma) and 200 µl of buffer (0.01 M of Tris-HCl [pH: 7.8] [10 mmol Tris-HCl], 0.005 M of EDTA [20 mmol EDTA], 0.5% SDS), and subsequent incubation at 40 °C overnight. Then protein content was extracted twice with 1 volume of alkali-phenol-chloroform-isoamyl alcohol (25:24:1). Finally, total DNA was precipitated by the addition of 1 volume of 2-propanol. Extracted nucleic acids were dissolved in 50 µl of TE buffer (pH 8.0).

PCR assay

HHV-6 and HHV-7: A nested PCR was performed for HHV-6 and HHV-7 as described previously (16). The HHV-6 sequences of outer primers were 5'ccgcaatcgaatccacctagcgg 3' and 5' gtgagaacggattcgaacagtgctg 3'. Inner primers were 5'cccatttacgatttcctgcaccacctc 3' and 5' ttcagggaccgttatgtcattgagcat 3'. The HHV-7 sequences of outer primers were 5 agttccagcactgcaatcg 3' and 5' cacaaaagctcgctatcaa 3'. Inner primers were 5' cgcatacaccaaccctactg 3' and 5' gactcattatggggatcgac 3'. Amplification reactions were performed in a final volume of 50 µl containing 10 mM of Tris-HCl (pH: 8.3), 50 mM of potassium chloride, 2.0 mM of magnesium chloride, 0.1% Triton X-100, 2.5 U of Taq polymerase (Boehringer Mannheim, Germany), 10 mM of deoxynucleoside triphosphates, 30 pmol of each outer primer and 10 µl of target DNA. The thermo was programmed first to preheat at 95 °C for 5 min to denaturate the samples and then the first round PCR reaction for HHV-6 was subjected to 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min in a DNA thermal cycler (MJ Research, Watertown, Massachusetts, USA). The HHV-7 DNA thermal condition included initial denaturation at 94 °C for 10 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s for 30 cycles and final extension at 72 °C for 5 min. The second round of PCR was performed as follows: 5 µl of the reaction mixture was transferred to the second round reaction mixture containing 30 pmol of each inner primer and the same buffer as in the first round. The temperature cycle of second round PCR was also the same. Multiple positive and negative controls were run in each nested PCR assay.

Fifteen microliters of the amplification products were analyzed by electrophoresis in an ethidium bromide stained 2% agarose gel and photographed under UV light by using Gel Doc 2000 (Bio-rad Laboratories-Segrate, Milan, Italy) (a 100 bp DNA ladder was used as a size marker to estimate the length of the products, BioLabs Inc.). The expected size of the nested PCR product for HHV-6 was 186 base pairs (bp) and the expected size of nested PCR product for HHV-7 was 264 bp.

CMV and EBV: The following primer pair was selected for CMV: 5' ttgcaggccacgaacaacgt 3' and 5' gtctacggattgctgacgct 3' (17). The sequence of the primers used was 5' ggc tgg tgt cac ctg tgt ta 3' and 5' cct tag gag gaa caa gtc cc 3' for EBV (18). The first round PCR procedures for CMV and EBV were performed as described above. The PCR mixture was subjected to initial denaturation at 94 °C for 5 min. Forty cycles of amplification were performed in a DNA thermal cycler. Each cycle consisted of a 1.5 min denaturation step at 94 °C, a 1 min annealing step at 60 °C, a 2 min extension step at 72 °C and a 5 min final extension step at 72 °C. Multiple positive and negative controls were run in each nested PCR assay.

Fifteen µl of the amplification products were analyzed by electrophoresis in an ethidium bromide stained 2% agarose gel and photographed under UV light by using Gel Doc 2000 (Bio-rad Laboratories-Segrate, Milan, Italy) (a 100 bp DNA ladder was used as a size marker to estimate the length of the products, BioLabs Inc.). The expected size of the PCR product for CMV was 305 bp

and the expected size of the nested PCR product for EBV was 239 bp.

To prevent contamination, pre-PCR and post-PCR steps were carried out in separate rooms. To detect carry-over contamination, negative controls were included in all reaction series: one negative sample was processed for every four to five tested specimens, and positive controls were included in each run. All PCR results were confirmed by repeating the entire PCR procedure.

Results

A total of 25 HSV-negative biopsy samples from 25 patients with EM were studied by PCR for the presence of CMV, EBV, HHV-6 and HHV-7 DNA sequences. The samples belonged to two males and 23 females. The age range was 29-53 years (mean: 40.08; median: 46.00). The disorder was recurrent in seven of 25 patients (28%). The patients neither had clinical/laboratory data of immunodefficiency, nor were they receiving immunosuppressive therapy.

By using the PCR, no positive signals for EBV, CMV, HHV-6 and HHV-7 were detected in any of the patient specimens (Figure).

Discussion

EM is thought to represent an immunologically mediated attack by cytotoxic T lymphoctes and/or NK cells and monocytes directed against keratinocytes expressing non-self, particularly viral antigens (3,4,7). HSV is the predominant virus in the herpesvirus family that has been linked to EM. By PCR, HSV DNA has been detected in one-third to two-thirds of specimens examined in several studies (5,8). The present study was based on the assumption that cases of EM that are HSV-negative might be provoked by infection with non-HSV members of the herpesvirus family.

CMV is an ubiquitous β-herpesvirus that can induce primary, latent, persistent and recurrent infections both in immunocompetent and immunodeficient individuals (12,19,20). Depending on the socioeconomic situation, anywhere from 40 to 100% of adults in a community can

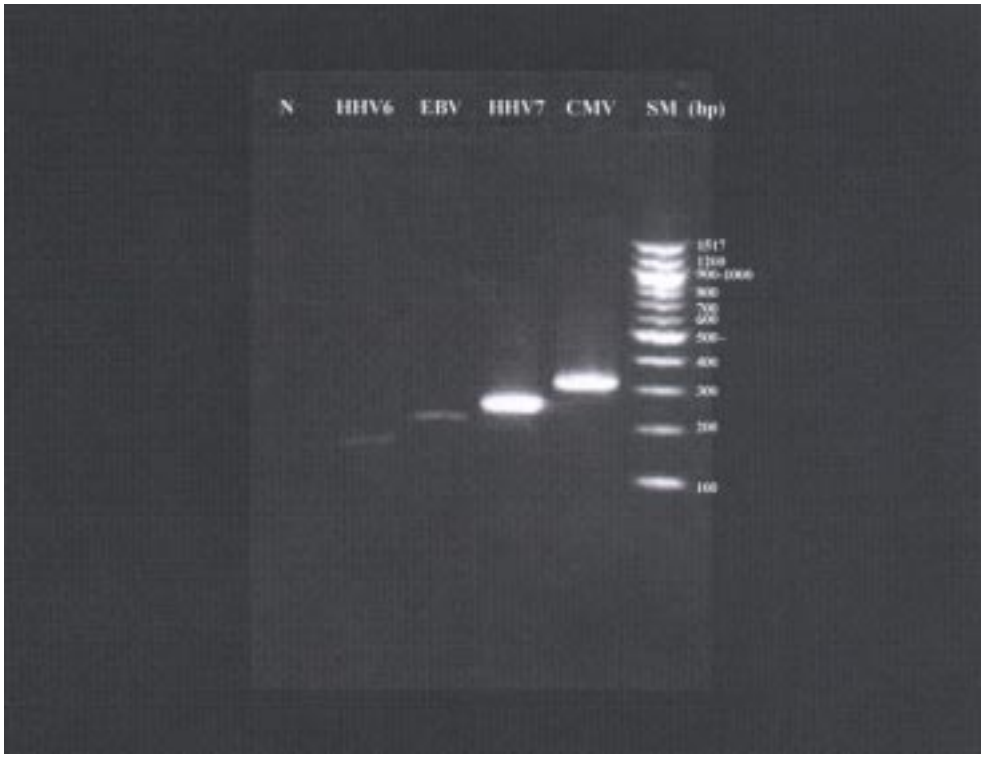

Figure: PCR amplification of CMV, EBV, HHV-6 and HHV-7 in lesional EM samples. *SM:* molecular DNA weight marker; *lane 1*: negative patient sample; *lanes 2,3,4,5*: positive control samples for HHV-6, EBV, HHV-7 and CMV respectively.

be infected with CMV (12,21). Ulceration of the perineum is the most common cutaneous finding of CMV infection (20). In the field of dermatology, CMV has been implicated in the etiopathogenesis of psoriasis, alopecia areata, vitiligo and SLE (20-22). Seishima et al. (23) suggested that CMV infection may lead to EM. The present study is the first study evaluating CMV-EM association that has failed to provide a supportive evidence of a role for CMV infection in EM.

EBV is an ubiquitous γ-herpesvirus that infects both adolescents and adults (12,24). The primary infection is infectious mononucleosis, followed by lifelong carriage of the virus in host tissues (4,24-26). EBV has been linked to several epithelial malignancies, lymphoproliferative disorders and lymphomas (24,25). EM has rarely been reported in EBV-associated disorders, including chronic fatigue syndrome (27). Chen et al. (4) investigated EM specimens by PCR and detected EBV-specific DNA sequences in two of 32 samples. However, the authors identified no EBER (EBV-encoded RNA) signal by ISH in the biopsy samples of any patient. The results of the present study are in accordance with those of Chen et al. and implicate that EBV does not play a major contributory role in the etiopathogenesis of EM.

HHV-6 is a β-herpesvirus similar to CMV (12). It was originally isolated in 1986 from peripheral blood leukocytes of patients with HIV infection and lymphoproliferative disorders (28). Primary infection is exanthem subitum (roseola infantum), following which the virus remains latent (12,29). HHV-6 is an ubiquitous virus and by the age of 1 year, 75% of infants have antibodies to HHV-6 (12). HHV-6 has been associated with lymphoproliferative disorders and lymphomas (13).

Finally, HHV-7 is a β-herpesvirus first isolated from human CD4 (+) T lymphocytes in 1990 (30). HHV-7 is distinct from but related to HHV-6 (12). The virus has been isolated from the peripheral blood of healthy people and has been reported in association with chronic fatigue syndrome, exanthem subitum-like illness and pityriasis rosea (12,16,29,31). The infection is very ubiquitous, and children are infected at an age similar to HHV-6 (12,29). Following primary infection the virus remains latent (29). To our knowledge, the association of HHV-6 or HHV-7 with EM has not been documented. The present study refutes a role for these viruses in EM.

The ability to amplify DNA and the sensitivity of PCR facilitates the detection of subpicogram amounts of pathogenic DNA sequences within host tissues (1,8,32). This exquisite sensitivity can lead to false-positive results, which represents a potential limitation of the diagnostic efficacy of the procedure (8). PCR can also give false-negative results due to inadequate sampling, lack of positive control specimens or presence of PCR inhibitors within the environment (33). In our study the risk of false- negative results has been decreased by the use of two sets of primers. In addition, positive control samples interspersed within the patient samples constantly gave positive signals for each virus and PCR procedures were repeated twice with reproducible results.

Herpesviruses are ubiquitous viruses with infection prevalences greater than that of EM prevalence. Given the absolute lack of PCR-positive cases in lesional EM specimens, we conclude that, except for HSV, the remaining herpesvirus family members can be excluded from the list of potential etiological factors in EM. Nevertheless, the role of other DNA or RNA viruses as initiators of HSV-negative EM is worth investigating and further studies are awaited.

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