Detection of Herpes Simplex Virus DNA in Cutaneous Lesions of Erythema Multiforme

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The association between erythema multiforme (EM) and herpes simplex virus (HSV) infection has long been appreciated, although the exact role which HSV may play in the pathogenesis of this herpes-associated EM (HAEM), is unknown. Previous studies have suggested, but not definitively demonstrated, the presence of HSV in lesions of HAEM. The presence of HSV would support the hypothesis that an immune-mediated response directed against HSV-specific antigens in the skin is central to lesion development in HAEM.

The purpose of this study was to examine lesions of EM for the presence of HSV DNA by using the polymerase chain reaction (PCR). In addition, in situ hybridization using an HSV-specific RNA probe was performed to further localize the HSV nucleic acids within the skin.

DNA was extracted from formalin-fixed, paraffin-embedded specimens of cutaneous lesions of HAEM and also from EM for which no precipitating factor could be documented, otherwise known as idiopathic EM (IPEM). DNA from lesions of bullous pemphigoid served as a negative control. Using PCR to specifically amplify HSV sequences which might be present, and then performing Southern analysis, we demonstrated HSV DNA in 9/13 HAEM and 6/9 IPEM biopsies. No HSV was detected in six lesions of bullous pemphigoid. In situ hybridization of three cutaneous HAEM lesions using an 35S-labeled HSV-specific RNA probe localized the HSV nucleic acids predominantly to the epidermis.

Three biopsies of chronic dermatitis, used as negative controls, did not demonstrate this specific hybridization. These findings confirm the presence of HSV in lesions of HAEM and are consistent with the concept of an HSV-specific immune-mediated pathogenesis for this disease. In addition, most cases of IPEM appear to be herpes associated despite the absence of clinically apparent HSV infection.


Erythema multiforme (EM) has captured the imagination of dermatologists for well over a century with its striking clinical presentation of symmetrically distributed, polymorphous lesions ranging from macules and papules to annular plaques, bullae and characteristic targets. Nevertheless, the pathogenesis of this acute, self-limited, often-recurrent disorder remains unclear. Likewise, the association of EM with an antecedent herpes simplex virus (HSV) infection has long been appreciated, but the mechanism by which HSV infection might lead to EM has yet to be defined.

EM has been classified as a hypersensitivity phenomenon or "reactive erythema," potentially precipitated by multiple factors, primarily drugs or infectious agents. Certainly the most common and best documented of these factors is HSV, both types 1 and 2.

Herpes-associated EM (HAEM) typically develops within three weeks following infection with HSV, and will often recur in these individuals with subsequent episodes of recurrent herpes infection. An estimated 15% to 63% of all cases of EM are secondary to HSV [1], and it has been suggested that most cases of so-called idiopathic EM (IPEM) are related to subclinical HSV infection [2,3].

Although there are several case reports of HSV being isolated from lesions of EM [4,5], viral cultures from EM lesions are usually negative. However, other indirect evidence for the role of HSV in the pathogenesis of HAEM includes the detection of HSV antigens in circulating immune complexes in patients with HAEM [6], viral particles seen by electron microscopy [4], and the demonstration of HSV-specific antigens in skin lesions of HAEM by immunofluorescence [7].

Recently, newer molecular biology techniques have made it possible to detect and to study specific nucleic-acid sequences which may be present in cells or tissue. One such technique is the polymerase chain reaction (PCR) [8]. Briefly, the PCR involves repeated cycles of three steps. The experimental DNA, referred to as the template, is combined with a thermostable DNA polymerase (Taq polymerase), nucleotides, and short oligonucleotide primers. The primers are selected and synthesized to be complimentary to the regions of the DNA template flanking the segment of interest. In the first step, the sample is denatured at a high temperature. This is followed by rapid cooling to allow the second step, annealing of the primers to occur. In the third step, the temperature is rapidly increased again for extension of the primers, resulting in replication of the DNA sequence of interest. These three steps are repeated multiple times, amplifying that portion of the template between and including the primer sites. Using the PCR, DNA which was initially present in minute quantities, may be specifically amplified to

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Reprint requests to: Sylvia L. Brice, M.D., Department of Dermatology, B-153, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Denver, CO 80262.
Abbreviations:
ACH: acetylcholine receptor
BP: bullous pemphigoid
EM: erythema multiforme
HAEM: herpes associated erythema multiforme
HSV: herpes simplex virus
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RAPID COMMUNICATION

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readily detectable levels amidst the milieu of cells in culture, or tissue specimens, even when formalin-fixed and paraffin-embedded [9,10].

The purpose of this study was to examine lesions of EM for the presence of HSV DNA using the PCR. In addition, in situ hybridization using an HSV-specific \(^{35}\)S RNA probe was performed to further localize the HSV nucleic acids within the skin.

**METHODS**

**Selection of Tissue** For this study, EM was defined by the following criteria: an acute, self-limited (less than six weeks), cutaneous or mucocutaneous illness; the presence of symmetrically distributed, discrete, round, fixed (lasting more than seven days), erythematous skin lesions; concentric color changes in at least some lesions (target lesions); and compatible histopathologic findings (primarily mononuclear cell infiltrate, no leukocytoclastic vasculitis). HAEM was defined as EM occurring within three weeks of a recurrent HSV infection. IPEM was defined as EM for which no precipitating factor could be determined, specifically no history of HSV infection or drug ingestion within three weeks. Biopsies were taken from cutaneous EM lesions, fixed in formalin, and embedded in paraffin. For the PCR, a total of 13 HAEM and 9 IPEM biopsies were studied. All HAEM biopsies, and 5/9 IPEM biopsies were obtained during episodes of recurrent disease. A confirmed history of recurrent disease was not available for the remaining four IPEM biopsies. Specimens utilized as controls included: six lesions of bullous pemphigoid, tissue infected with HSV-1 and HSV-2, and tissue infected with other herpes viruses (varicella-zoster virus, cytomegalovirus, and Epstein-Barr virus). Formalin-fixed, paraffin-embedded biopsies were also utilized for in situ hybridization and included three lesions of HAEM, three lesions of chronic dermatitis and one lesion of herpes labialis.

**Extraction of DNA from Tissue for Use in PCR** DNA was extracted from each tissue block, as previously described [9,10] with minor modifications. Twenty-five 5-um sections were cut from each block, de-paraffinized in xylene, and rinsed with 95% ethanol. After drying, the tissue pellets were resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.8) and incubated with Proteinase K overnight at 37°C. The samples were incubated for ten minutes at 95°C, spun, and the supernatants retained for use in the PCR.

**The Polymerase Chain Reaction** Twenty-two base oligonucleotide primers which flank and amplify a 92 base pair portion of the HSV genome coding for DNA polymerase were synthesized. These primers were previously demonstrated to be specific for HSV-1 and HSV-2, with no cross-reactivity with other herpesviruses or with mammalian DNA [10]. Since this was the anti-sense transcript, both mRNA and DNA could potentially be detected. The probe was suspended in hybridization buffer (50,000 cpm/20 ul), placed over the tissue sections, and covered by glass coverslips sealed with rubber cement. The probe and tissue sections were denatured at 95°C for 15 min, and hybridization continued overnight at 42°C. The slides were washed to a maximum stringency of 0.5X SSC, 1 mM EDTA, 2.5 uM DTT at 68°C, dipped in Kodak NTB-2 emulsion, exposed for two to three weeks and then developed. Sections were stained with hematoxylin and eosin and examined by light microscopy. As an additional negative control, a second \(^{35}\)S-labeled RNA probe corresponding to a portion of a rat acetylcholine receptor (ACH) gene was used for in situ hybridization with each of these tissue specimens. This ACH probe was comparable in size and specific activity to the HSV probe.

**RESULTS**

**Detection of Amplified DNA** On visualizing the DNA on the gels with ethidium bromide, bands of the anticipated size for the amplified HSV and BG fragments were readily detected, although in some cases background bands made conclusive interpretation difficult. However, by Southern analysis with the labeled internal oligonucleotides, either no signal was seen or a single, unambiguous band in the anticipated location for each primer was seen in each lane. These results are given in Table I and representative specimens illustrated in Figure 1. Amplification with the BG primers occurred with all specimens. Amplification with the HSV primers was seen in 9/13 HAEM and 6/9 IPEM. Fifty percent of the HSV positive IPEM biopsies were obtained from patients with known recurrent disease, and 50% from IPEM patients for whom a confirmed history of recurrence was not available. Not included within these results or in Table I is a second biopsy from one patient with recurrent IPEM, obtained during a subsequent episode of EM. HSV was detected in both biopsies from this patient. A very strong amplification was seen for both HSV-1 and HSV-2 with the HSV primers. No amplification was seen with the HSV primers for the bullous pemphigoid lesions or tissue infected with other herpesviruses.

<table>
<thead>
<tr>
<th>Tissue Tested</th>
<th>HSV Positive/Total</th>
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<tbody>
<tr>
<td>HAEM</td>
<td>9/13 (69%)</td>
</tr>
<tr>
<td>IPEM</td>
<td>6/9 (67%)</td>
</tr>
<tr>
<td>BP</td>
<td>0/6 (0%)</td>
</tr>
</tbody>
</table>
Figure 1. Southern analysis of DNA amplified by the PCR. DNA in the first lane of each pair was amplified with the HSV primers, while DNA in the second lane of each pair was amplified with the BG primers. Using $^{32}$P-labeled internal oligonucleotides to detect the amplified DNA, BG is demonstrated in all specimens. HSV DNA is demonstrated within lesions of herpes labialis (HSV), HAEM and IPEM, but not within BP.

In Situ Hybridization

Hybridization of the HSV probe was detected primarily within the epidermis in herpes labialis and all three lesions of HAEM. In each case, diffuse epidermal staining, as well as focal areas of what appeared to be perinuclear accentuation were appreciated. These two patterns of staining are illustrated in Figure 2. Within the lesions of chronic dermatitis, slight background binding was present uniformly over the specimens, but no specific hybridization with the HSV probe was detected (Fig 2C). Using the ACH probe, no specific hybridization was seen with any of the tissue specimens (not shown).

DISCUSSION

HAEM is by far the most-common subset of EM, occurring primarily in young, healthy adolescents and adults, the age group in which frequent recurrences of HSV would be anticipated. HAEM is almost always manifested as EM minor [2], namely cutaneous lesions of several weeks' duration, with limited mucosal, usually oral, involvement. The lesions typically develop from three to 21 days following herpes infection, and this time delay appears to be quite constant for each individual [1]. Recurrences are frequent, although they do not necessarily follow each episode of herpes infection, nor does a clinically apparent episode of herpes always antedate the EM.

Bateman is given credit for being the first to recognize an association between HSV and EM as early as 1813 [15]. Since that time, support for this association, based on clinical grounds, has been well-established [2]. Currently, the efficacy of acyclovir in suppressing lesions of recurrent HSV and the subsequent EM [16] is further evidence of an etiologic relationship. However, exactly what role HSV plays in the pathogenesis of HAEM remains unclear. Attempts to demonstrate conclusively HSV in HAEM lesions have met with little success. Viral cultures from HAEM have been almost uniformly negative. In the few cases where HSV isolation has been reported [4,5], the patients were generally immunocompromised with extensive cutaneous disease. An individual instance in which viral particles were detected within HAEM lesions by electron microscopy has been reported [4]. Recently, Orton et al [7] provided the most compelling evidence to date by using indirect immunofluorescence to demonstrate the presence of HSV-specific antigens within the epidermis of HAEM lesions.

Despite these findings, the pathogenesis of EM, including HAEM, remains a matter of speculation, although immune mediated mechanisms are almost certainly involved. Most evidence

Figure 2. In situ hybridization using the HSV-specific RNA probe. The epidermis of a lesion of herpes labialis shows specific hybridization with some increase overlying nuclei (A). A more diffuse pattern of specific hybridization is seen within the epidermis of a lesion of HAEM (B). No specific hybridization is detected in a lesion of chronic dermatitis (C).
promised patients. A recent study demonstrating a strong association of HAEM, but not recurrent HSV alone, with the HLA DQw3 suggests a possible HLA related altered immune response to HSV in individuals who develop HAEM [3]. The answers to the questions posed here are, of course, only speculative, and additional investigation will be necessary to confirm or refute their validity. Of particular importance will be to determine whether or not HSV is present in the peripheral blood cells of HAEM patients on a continuous or sporadic basis, and whether HSV can be detected in normal skin of HAEM patients during an active episode, or in previously involved skin between active episodes of HAEM.

In summary, in this study we have demonstrated the presence of HSV DNA within cutaneous lesions of HAEM and IPEM, and the HSV appears to be in a predominantly epidermal location. These findings are consistent with the premise that an immune mediated response directed against HSV present in the epidermis is involved in the pathogenesis of HAEM. In addition, most cases of "idiopathic" EM appear to be herpes associated despite the absence of clinically apparent HSV infection.

We gratefully acknowledge the assistance of Dr. Jay Jester and Dr. Loren Golitz in acquiring the biopsies of patients included in this study, and Ms. Kris Olson for instruction in performing the in situ hybridization.

REFERENCES