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Herpes Simplex Virus Antigens and Inflammatory Cells in Oral Lesions in Recurrent Erythema Multiforme

Immunoperoxidase and Autoradiographic Studies

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Herpes simplex virus (HSV) antigens were sought in 15 biopsy specimens from both lesional mucosa and clinically healthy looking oral mucosa between attacks in patients with erythema multiforme (EM). Four of the eight biopsy specimens obtained from lesional EM mucosa stained positively with HSV-1- and/or HSV-2-specific antisera applied in direct immunoperoxidase staining. Of the 16 tissue specimens used as controls, two displayed positive staining with HSV-1 and/or HSV-2. Five of the seven biopsy specimens from macroscopically healthy oral mucosa obtained between attacks from patients with recurrent EM stained positively with HSV-1 and/or HSV-2. Of the six tissue specimens used as controls, three stained positively. Most of the local inflammatory mononuclear cells belonged to the T cell series, mainly to the CD-4 subset. A small proportion of the local T cells were blast transformed as assessed by CD-25 expression and [^{3}H]thymidine incorporation. This, together with the findings showing a lower degree of activation in the biopsy from macroscopically healthy looking mucosa between attacks suggest an active role of the cell-mediated immune response in the genesis of oral lesions in EM. The persistence of HSV antigens, and the well-established role of HSV as a precipitating factor in recurrent EM, suggest that HSV may be involved, but since HSV seems to be present in other mucosal lesions as well as in clinically healthy mucosa, quite frequently an additional, hitherto unknown factor must be present in order that EM may occur. ***Key word: T cells.***

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Erythema multiforme (EM) is an acute, vesiculobullous, self-limiting disease of the skin and mucous membranes (1). Although the etiology of EM is obscure, the association of recurrent herpes simplex virus (HSV) infections with EM is widely discussed in the literature (1, 2–4). EM presents a varied picture both clinically and histologically. In skin lesions, inflammatory mononuclear cells are abundant (5–7), though their degree of activation is largely unknown in oral lesions.

The aim of the present study was to elucidate whether HSV is present more frequently in the oral lesional mucosa and/or in healthy looking oral mucosa between EM attacks in patients with EM than in other mucosal lesions or clinically healthy looking mucosa in otherwise healthy persons. The phenotype and activation of mononuclear cells were studied to determine whether an immunologic reaction might be implicated.

PATIENTS AND METHODS

Patients

Twenty-one biopsy specimens were obtained under local anesthesia from 18 patients with EM. Ten of these specimens were from oral lesions in the same number of patients. In 3 of the 10 patients, biopsies were also taken from clinically healthy looking mucosa between attacks. An additional eight specimens were taken between attacks from the macroscopically healthy looking mucosa of 8 patients with a previous history of recurrent EM. None of the patients were receiving systemic medication at the time of biopsy. Patient no. 10 had received orally administered acyclovir prior to biopsy, which was performed on macroscopically healthy looking oral mucosa. The clinical and histologic criteria for the diagnosis of EM were those proposed by Huff et al. (1) and each patient fulfilled these criteria (Table I).

Controls for the immunoperoxidase staining of lesional EM mucosa for HSV-1 and HSV-2 antigens were oral biopsy specimens taken from a total of 16 patients: 13 with oral lichen ruber planus, 2 with recurrent oral ulcers and one with benign mucous membrane pemphigoid. Controls

Table I. *Studies performed on 21 biopsy specimens from 18 patients with EM*

Pat. no.	Site of biopsy	IP	MCD	LAM	Etiologic factor
Lesional mucosa					
1.	Buccal	–	×	×	?
2.	Gingiva	×	×	×	?
3.	Lip	–	×	×	?
4.	Gingiva	×	×	×	HSV
5.	Buccal	×	–	–	?
6.	Palatal	×	–	–	HSV
7.	Lingual	×	–	–	Drug
8.	Buccal	×	×	×	HSV
9.	Buccal	×	–	–	?
10.	Buccal	×	–	–	HSV
Clinically healthy oral mucosa between EM attacks					
8.	Buccal	×	–	–	HSV
9.	Buccal	–	×	–	?
10.	Buccal	×	×	–	HSV
11.	Buccal	–	×	×	HSV
12.	Buccal	×	×	×	HSV
13.	Buccal	×	×	–	HSV
14.	Buccal	–	×	×	HSV
15.	Buccal	×	×	–	HSV
16.	Buccal	–	×	×	HSV
17.	Buccal	×	×	×	HSV
18.	Buccal	×	–	–	HSV

IP = immunoperoxidase staining for HSV
MCD = mononuclear cell differentation
LAM = lymphocyte activation marker analysis
HSV = herpes simplex virus

for clinically healthy looking mucosa in EM were oral biopsy specimens taken from 6 patients with a clinically healthy buccal mucosa and no history of dermatologic or mucosal disease.

Informed consent was obtained from all patients before enrolling them in the study.

Methods

Immunoperoxidase staining for HSV antigens. The formalin-fixed and paraffin-embedded biopsy specimens were prosessed for direct immunoperoxidase staining for HSV antigens by using undiluted affinity-purified rabbit anti-HSV type 1 and type 2 horseradish peroxidase-conjugated antiserum (P175, P176, Dakopatts a/s, Copenhagen, Denmark). The method used is described in detail elsewhere (8). DAKO Control slides (T1150, Dakopatts) were used as positive controls for HSV antigen.

For detection of HSV antigens, the entire specimen was examined. The epithelial cells were considered HSV-positive if the nucleus stained clearly brown. Since HSV-1 and HSV-2 have type-specific as well as type-common determinants, no distinction between these two was made in the present study.

Isolation of HSV from viral cultures of oral lesions, fluorescent antibody tests and complement fixing antibody titres from serum samples were estimated in patients and in controls.

Immunohistochemistry. Immunohistochemically the phenotype and activation marker expression of B and T lymphocytes were studied by using monoclonal antibodies in the avidin–biotin–peroxidase complex (ABC) method on cryostat sections. Following monoclonal antibodies were used: CD-2, CD-4, CD-8, CD-15, MHC locus II antigen (= Ia) (Ortho Diagnostic System Inc., Raritan, NJ, USA), CD-19, CD-25 (= Tac) (Dakopatts, Copenhagen) and PCA-1 (Coulter Immunology, Hialeah, Fla., USA). The inflammatory cells were counted using an ocular containing square (20 squares × 20 squares) and oil immersion objective (×1000 magnification). Exogenous peroxidase positive, specifically stained cells were clearly discerned by brown staining of the membrane and were thus readily distinguishable from unstained cells under a light microscope. At least 200 inflammatory cells were counted in each specimen.

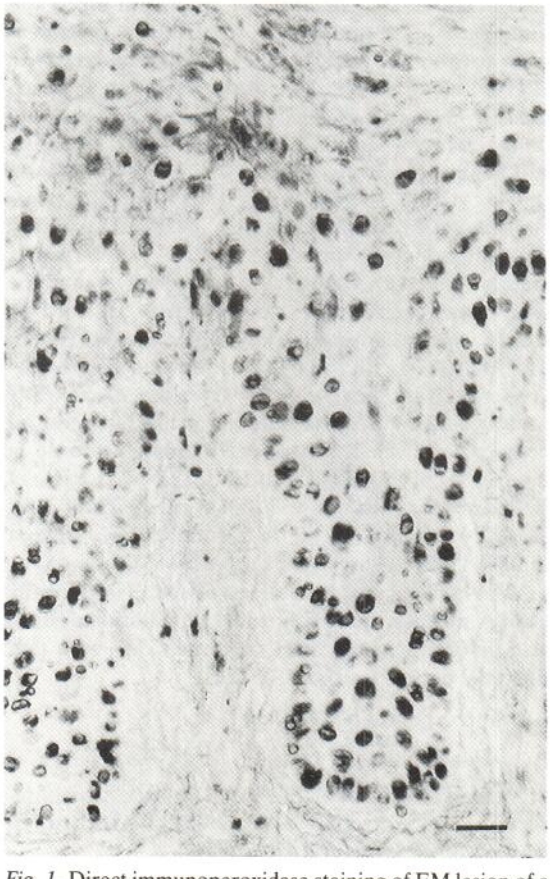

Fig. 1. Direct immunoperoxidase staining of EM lesion of a patient with recurrent EM. Nuclei of oral mucosal cells containing HSV-1 antigen as revealed by direct immunoperoxidase staining with affinity-purified anti-HSV-1 antiserum. ×480. Bar = 20 μm.

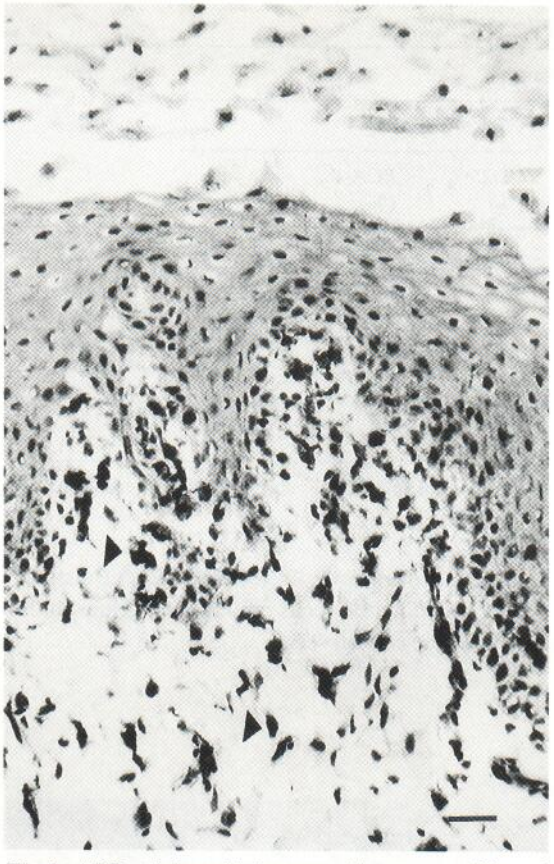

Fig. 2. ABC staining with hematoxylin counterstaining of apparently macroscopically normal buccal mucosa obtained between attacks from a patient with recurrent EM lesions. CD-15-positive mononuclear phagocytes *(arrowheads)* in the upper subepithelial connective tissue layer. CD-15-positive cells are located near the capillaries in the subepithelial connective tissue layer, just below the basement membrane. ×312. Bar = 30 μm.

Autoradiography-ABC double labelling. DNA synthesis of B and T lymphocytes was studied by autoradiography-ABC double labelling. After incorporation of [^{3}H]thymidine and ABC staining; some CD-2, CD-19 and PCA-1 stained slides were further prosessed for autoradiography.

The methods used are described in detail elsewhere (9).

RESULTS

Direct immunoperoxidase staining for HSV-1 and HSV-2

Immunoperoxidase staining with HSV-specific antiserum showed positive staining for HSV antigens in the nuclei in a total of four out of eight biopsy specimens obtained from the EM lesions of 8 patients. Of the four biopsy specimens that stained positively for HSV antigens, three were from patients suffering from herpes associated erythema multiforme (HAEM). Of the four biopsy specimens in which no HSV antigens were detected, only one was from a patient with HAEM (Table I). Only two of the 16 control specimens (13%) from patients with other mucosal diseases showed positive staining for HSV antigens. The difference between these groups is statistically slightly significant ($X^2 = 5.06$, 1 DF, $p < 0.05$), (Fig. 1).

Five of seven biopsy specimens (71%) obtained between attacks from the macroscopically healthy oral mucosa of 7 patients with recurrent HAEM showed positive staining for HSV antigens. Three of the six control specimens showed positive staining

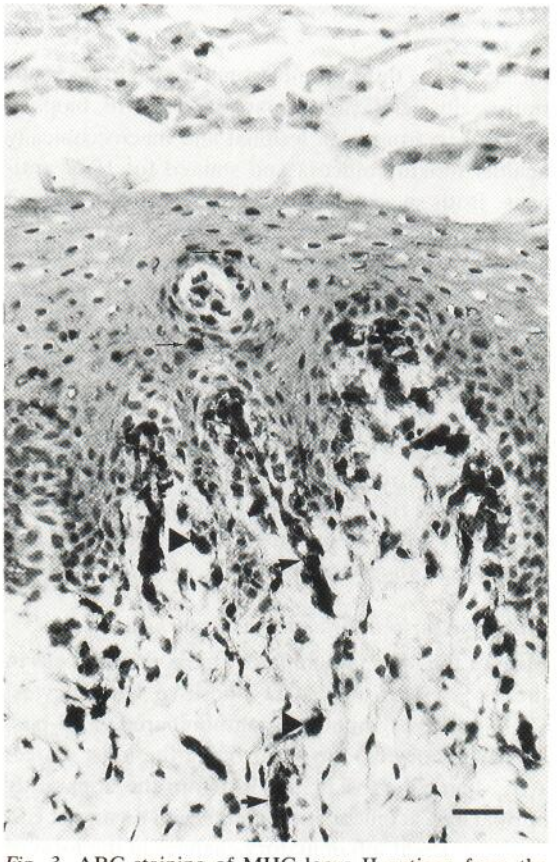

Fig. 3. ABC staining of MHC locus II antigen from the same patient as in Fig. 2. MHC locus II antigen positive cells are visible *(arrowheads)* in the same location as CD-15-positive cells. Endothelial cells in the capillaries *(large arrows)* and small blood vessels (not shown) also display MHC locus II antigen. In addition, some cells in the epithelial cell layer are MHC locus II antigen positive *(small arrows)*. Hematoxylin counterstaining, ×312. Bar = 30 μm.

Table II. *Lymphocyte activation markers in situ in patients with EM*

Pat. no.	Mucosa						Pat. no.
	Lesional			Clinically healthy			
	CD25 (%)	PCA-1 (%)	Ia (%)	CD25 (%)	PCA-1 (%)	Ia (%)	
1.	0.6	19	79	0	0	73	11
2.	2.5	3	75	0	0	78	12
3.	3.6	12	89	0	2	82	14
4.	0.4	11	71	0	0	65	16
8.	0.6	14	81	0	0	75	17
X	1.5	12	79	0	0.4	75	
SEM	0.6	2.6	3	0	0.4	2.8	

The Mann-Whitney U- test gave $p < 0.005$ for the difference in CD25 (= Tac) and PCA-1 values, but the difference for the Ia values was not significant.

for HSV. The difference between these groups is not statistically significant. In patients 8 and 10, biopsies were taken from both lesional and macroscopically healthy looking mucosa and stained for HSV antigens. Both specimens taken from patient 8 stained positively for HSV, while in patient 10, HSV antigens were detected only in the lesional mucosa. This patient was treated with systemic acyclovir prior to the second biopsy.

All Dako control slides which were used as positive controls stained positively.

In none of the patients with recurrent EM could HSV be demonstrated by isolation or fluorescent antibody test from viral cultures of oral EM lesions. Complement fixing antibody titres against HSV varied from 0 to 64 in patients as well as controls and were thus within normal limits.

Mononuclear cell differentiation markers

Most of the local inflammatory mononuclear cells in the EM lesions were CD-2-positive lymphocytes. CD-4-positive lymphocytes outnumbered CD-8-positive lymphocytes, the latter being the most scarce. ABC stained cryostat sections from the apparently normal buccal mucosa of patients with recurrent EM disclosed slight inflammatory changes in the form of predominantly CD-l5 and MHC locus II antigen positive mononuclear cells (Ia cells) in the papillary subepithelial connective tissue layer (Figs. 2, 3). Moreover, occasional T lymphocytes, mostly belonging to the CD-8 subset, were seen in the papillary subepithelial connective tissue and in the epithelium. Accordingly, EM lesions and macroscopically healthy looking mucosa showed a totally different distribution of inflammatory cells among the various major mononuclear cell subsets.

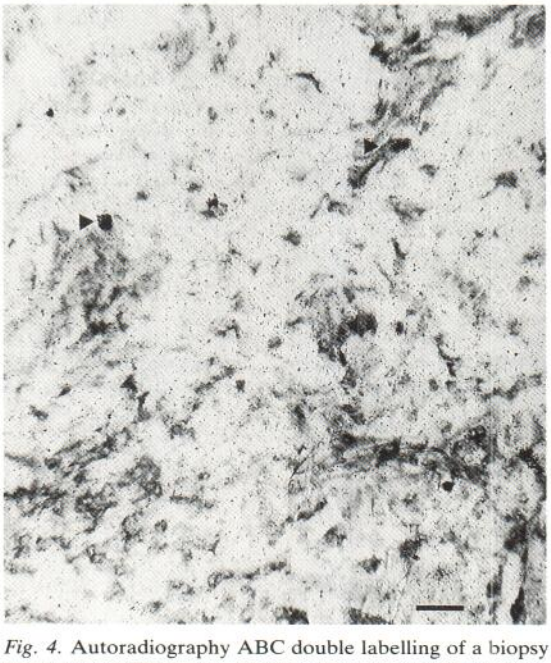

Fig. 4. Autoradiography ABC double labelling of a biopsy from an oral EM lesion. Despite the relatively large number of cells with CD4 phenotype, only occasional [^{3}H]thymidine incorporation blasts *(arrowheads)* are seen in the inflammatory mononuclear cell infiltrate. ×312. Bar = 30 μm.

Mononuclear cell activation markers and DNA synthesis

ABC staining of the CD-25 receptor, PCA-1 epitope and MHC locus II coded Ia antigen was used to visualize cells past the G0/G1 interphase. In general, cells carrying the CD-25 receptor or PCA-1 epitope were relatively few in EM lesions (Table II).

However, the relative proportion of CD-25- and PCA-1-positive cells was greater in lesions than in healthy looking mucosa. In contrast, there was no difference in MHC locus II antigen expression between lesions and the mucosa between attacks (Table II).

[^{3}H]thymidine incorporation with subsequent autoradiography was used to visualize cells in the S phase of the cell cycle. DNA synthesis in basal epithelial cells showed that the radioactive label had penetrated deeply into the sample. However, less than 1% of all inflammatory mononuclear cells in the EM lesions had incorporated the labelled thymidine. Most of the S-phase cells had a CD-2/CD-4 phenotype, as shown by our double labelling method (Fig. 4).

In the macroscopically healthy looking buccal mucosa of patients with recurrent EM, occasional S-phase blast cells, mostly from the CD-8 subset, were found in the sparse inflammatory cell infiltrate in the papillary subepithelial connective tissue layer or in the epithelium.

DISCUSSION

Our findings show evidence of the persistent HSV antigens in oral mucosal cells in most patients with recurrent EM, even in the absence of positive results from more conventional tests. Moreover, EM lesions were characterized by a T-cell-rich mononuclear cell infiltrate, which is consistent with our findings in transmission electron microscopy (TEM) (10). It is therefore tempting to speculate on a role for a virus antigen-induced cell-mediated immune response as a possible mechanism involved in the etiopathogenesis of this disease. However, most patients with HSV infection do not develop EM, and HSV seems to be present in other mucosal lesions as well as quite frequently in clinically healthy mucosa. Therefore other factors, such as inherited susceptibility, may be involved in those EM patients showing HSV in their healthy or lesional mucosa. On the other hand, persistence of HSV antigens (or closely related immunoreactive structures), as well as the well established role of HSV as a precipitating factor, both suggest some kind of connection. This supports and further elucidates earlier observations on the presence of HSV antigen in skin lesions in HAEM (11).

Most of the inflammatory mononuclear cells in oral EM lesions were T lymphocytes which, due to their role in cell-mediated immune reactions, may play a role in the etiology of lesions. However, T cells past the G0/G1 interphase or in the S phase of the cell cycle, assessed by CD-25 receptor expression and [^{3}H]thymidine incorporation, were relatively few. In contrast to the above-mentioned evidence which favours blast cell transformation on only a limited scale, MHC locus II antigen was expressed by most of the local inflammatory mononuclear cells. Obviously a great many of these cells belong to the T cell series, by virtue of overlap between MHC locus II antigen and T cells, although a proportion definitely represent other cells such as monocytes, dendritic cells, plasma cells, or even some resident cells such as endothelial cells and tissue macrophages. This might be due to gamma-interferon secretion by the few activated T blasts. Gamma-interferon is known for its ability to induce MHC locus II antigen expression in concentrations that are so low that they do not have the better known antiproliferative or anti-viral effects (12). Because of the role of endogenous MHC locus II antigen presentation and immune induction, this might be a significant factor in the development of oral EM lesions. This is also supported by our finding of MHC locus antigen-positive cells during attacks in many patients with recurrent EM.

In conclusion, our findings suggest that hidden HSV antigens and the cell-mediated immune response may play an important role in the pathogenesis of EM, but quite obviously an additional factor is needed in order for EM to occur.

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