

B- and T-LYMPHOCYTE DISTRIBUTION IN BENIGN AND MALIGNANT LYMPHOEPITHELIAL LESIONS OF THE PAROTID GLAND: CORRELATION WITH EPSTEIN-BARR VIRUS EXPRESSION AND A PROPOSED MECHANISM OF MALIGNANT TRANSFORMATION

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Epstein-Barr virus expression in malignant lymphoepithelial lesions (LEL) of the parotid gland has been well established. The virus is occasionally expressed in benign LEL, especially in immunocompromised hosts. The pathogenesis of the disease as it relates to virus expression and lymphocyte subsets has not been clearly defined. In this study, we attempted to identify B- and T-lymphocyte distribution in the lesions as it relates to EBV expression in LELs of the parotid gland. Formalin-fixed paraffin-embedded sections of 18 cases of LEL of the parotid gland were immunohistochemically tested for the distribution of B- and T-lymphocytes in the lesions, using the antibodies L-26 (CD 20) for B-lymphocytes and UCHL-1 (CD-45RO) for T-lymphocytes. The sections were also tested by *in situ* hybridization for EBV mRNA expression, using the EBER-1 probe specific for EBV-1 gene. The 18 lesions included seven malignant LEL, seven benign LEL and four benign lymphoepithelial cysts. All malignant LELs showed a high and diffuse level of epithelial expression of EBV mRNA. Of the 11 benign lesions, only one case showed focal epithelial expression of EBV mRNA. This was a case of benign LEL in an HIV-positive male. All the benign lesions, except that expressing EBV mRNA, showed a T-/B-lymphocyte ratio averaging 2:1. All cases expressing EBV mRNA, including the case of benign LEL in the HIV-positive patient, showed a T-/B-lymphocyte ratio averaging 1:3. Our findings suggest that a T-lymphocyte-mediated immune response may play an essential role in suppressing proliferation of EBV in benign LEL of the parotid gland. This immune mechanism may be significantly disturbed in the malignant lesions, leading to uncontrolled viral replication and carcinogenesis. *Ann Saudi Med* 1997;17(1):4-9.

Malignant lymphoepithelial lesions (MLELs) of the parotid gland were first described by Hilderman et al. in 1962.¹ The tumors are poorly differentiated squamous carcinomas with a prominent lymphocyte-rich stroma.² The tumors show an increased incidence among Canadian, Alaskan, and Greenland Eskimos³ (or Inuits, as they are known in Canada). The association of the neoplasm with Epstein-Barr virus (EBV) has been well established.⁴⁻⁸ The pathogenesis of the neoplasms as it relates to the EBV is, however, not clear. The relationship of the neoplasm with the immune system as it relates to EBV viral expression has not been fully characterized.

Benign lymphoepithelial lesions (BLEL) are known to coexist with MLEL.⁹ Benign lymphoepithelial cysts (BLEC) are also known to coexist with BLEL, which suggests a pathogenetic spectrum.¹⁰ BLEC show an

increased incidence in HIV-positive patients, which suggests an underlying immune mechanism involved in the pathogenesis of these lesions.¹¹⁻¹⁶ EBV has been identified by polymerase chain reaction (PCR) in benign salivary glands, including those showing BLEL.⁸

In the current study, we tested the EBER-1 probe specific for EBV-1 gene and its capability to detect the virus in benign and malignant LEL. We also performed immunohistochemical studies to identify T- and B-lymphocyte distribution in the lesions and their ratios. We hoped the study would shed some light on the pathogenesis of malignancy as it relates to EBV expression and the patients' immune system in relation to EBV expression in benign and malignant lesions.

Material and Methods

Eighteen cases of LELs of the parotid gland formed the basis for this study. The cases included seven cases of MLEL, seven cases of BLEL, and four cases of benign lymphoepithelial cysts. The cases were obtained from the files of the University of Alberta and of King Faisal Specialist Hospital and Research Centre. The slides were

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reviewed and the diagnosis of MLEL was confirmed according to the criteria first described by Hilderman.¹ The lesions should have a lymphocyte-rich stroma with the occasional finding of adjacent benign lymphoepithelial lesions. BLEL were diagnosed according to criteria set by the World Health Organization.¹⁷ The lesions should have the characteristic epimyoeplithelial islands with a lymphocyte-rich stroma. Benign lymphoepithelial cysts were diagnosed according to the criteria outlined in the literature.¹¹⁻¹⁶

EBV In Situ Hybridization

Representative four-micron sections of the formalin-fixed paraffin-embedded tissue of each lesion and control were obtained and mounted on aptex-coated slides. *In situ* hybridization was performed using a 30 base oligonucleotide probe (5'-AGA CAC CGT CCT CAC



FIGURE 1. Benign lymphoepithelial lesion showing the characteristic epimyoeplithelial islands with lymphocyte-rich stroma (Hematoxylin and eosin, 160x).

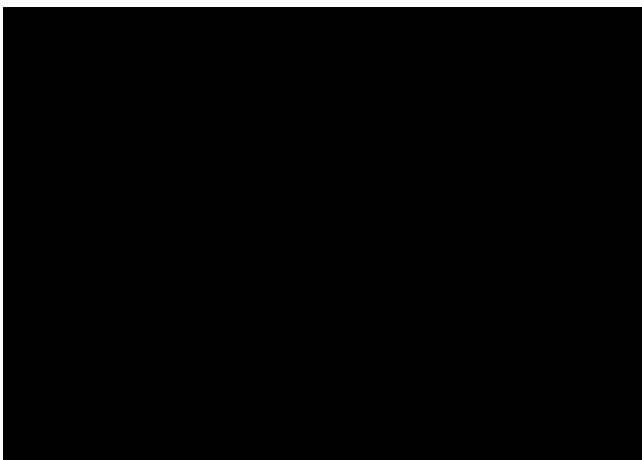


FIGURE 2A. MLEL showing anaplastic cells with rich lymphocytic stroma (Hematoxylin and eosin, 160x).

CAC CCG GGA CTT GTA-3') complementary to a region of the EBER-1 gene of EBV-base pairs 69-98. The probe was labeled with digoxigenin and *in situ* hybridization was done following procedures already outlined in the literature.⁴ Any level of viral expression detected in the epithelial or lymphoid cells was considered positive for viral expression. Twenty cases of benign and malignant nonlymphoepithelial lesions of the parotid gland were used as controls. Positive controls of known EBV-positive cases of nasopharyngeal carcinoma were used with each run as positive controls. A nonsense probe (human papilloma virus) was also used with each run as a negative control.

Immunohistochemical Identification of T- and B-Lymphocytes

Unstained sections of the benign and malignant lesions were immunostained for T- and B-lymphocyte distribution. The immunoperoxidase technique introduced by Hsu et al. was used.¹⁸ The UCHL-1 (CD45RO) antibody was used to identify an antigen seen in primed mature T-lymphocytes.¹⁹⁻²¹ The L-26 (CD20) antibody was used to identify an antigen seen on the surface of most mature B-lymphocytes.²² A rough estimate of T- to B-lymphocyte ratios was performed in the benign and malignant lesions. The estimation was performed in the lymphocytes seen in the regions of the benign and malignant lymphoepithelial lesions.

Results

The 18 LELs were seen in nine male and nine female patients. The male-to-female ratio in the benign lesions was 3:8, whereas MLELs were exclusively seen in males. The ages ranged from 20 to 87 years (median 42 years), with a similar age distribution in the benign and malignant lesions. All patients with MLEL, except one, were native

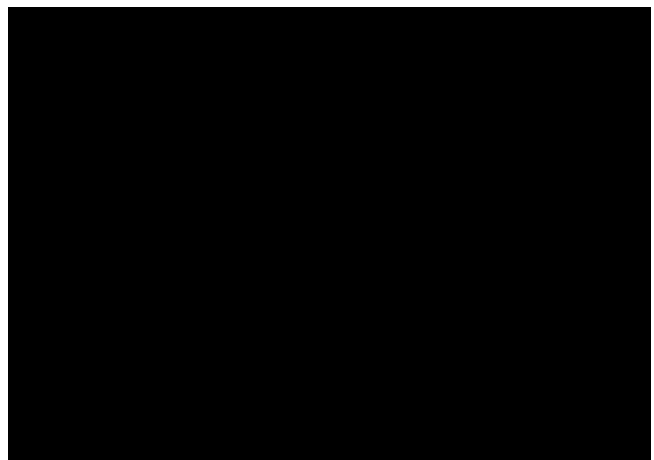


FIGURE 2B. MLEL showing strong and diffuse EBV mRNA expression in the epithelial cells (*in situ* hybridization EBER-1 probe, 40x).

TABLE 1. Clinical and pathological findings relevant to the 18 patients studied and their EBV mRNA expression profiles.

Patient	Age	Sex	Race	Diagnosis	EBV mRNA	T-/B-lymphocyte ratios
1	39	M	Inuit	MLEL	+++	1/3
2	44	M	Inuit	MLEL	+++	1/4
3	42	M	Inuit	MLEL	+++	1/2
4	45	M	Inuit	MLEL	+++	1/4
5	40	M	Inuit	MLEL	+++	1/6
6	47	M	Inuit	MLEL	+++	1/3
7	53	M	Caucasian	MLEL	+++	1/3
8	20	M	Caucasian	BLEL	-	1/1
9	36	M*	Caucasian	BLEL	+	1/4
10	40	F	Caucasian	BLEC	-	2/1
11	40	M	Caucasian	BLEC	-	2/1
12	47	F	Inuit	BLEC	-	1/1
13	50	F	Caucasian	BLEL	-	3/1
14	53	F	Inuit	BLEC	-	1/1
15	23	F	Chinese	BLEL	-	1/1
16	87	F	Caucasian	BLEL	-	2/1
17	31	F	Caucasian	BLEL	-	2/1
18	45	F	Caucasian	BLEL	-	2/1

*HIV-positive male; MLEL=malignant lymphoepithelial lesion; BLEL=benign lymphoepithelial lesion; BLEC=benign lymphoepithelial cyst.

Canadian (Inuit), whereas the majority of the patients with BLEL were Caucasian, with only two Inuit patients and one Chinese patient. The benign lesions showed the characteristic epimyoeplithelial islands with a lymphocyte-rich stroma (Figure 1). The malignant lesions showed islands of anaplastic or squamous carcinoma surrounded by benign-appearing lymphocytes (Figure 2).

EBV Expression

None of the control specimens showed any viral expression. All the malignant lesions showed diffuse and intense expression of EBV viral mRNA. The expression was seen in the epithelial cells and not in the adjoining lymphocytes or benign lymphoepithelial lesions (Figure 2). None of the benign lesions showed any evidence of EBV expression, except one case of BLEL. The case was seen in a 36-year-old HIV-positive white male. The level of expression was focal and less intense. The expression was also seen in the epithelium and not in the lymphocytes (Figure 3).

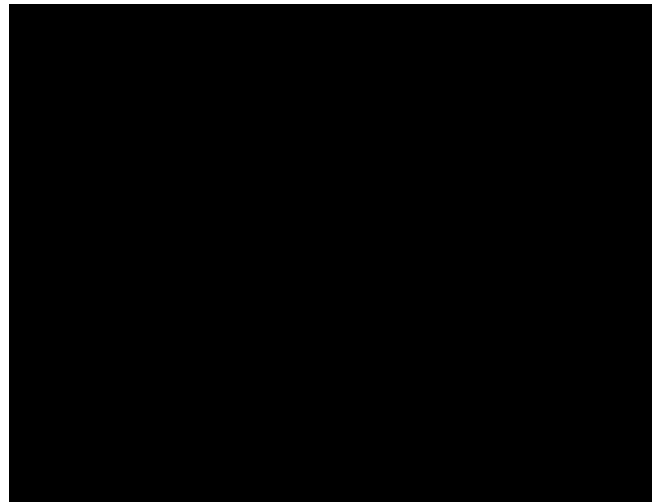


FIGURE 3. BLEL in a 36-year old HIV-positive male showing benign lymphoepithelial islands among local EBV mRNA expression (*in situ* hybridization EBER-1 probe, 100x).

T- and B-Lymphocyte Ratios

All BLEL, except for the lesion in the HIV-positive patient, showed a T-/B-lymphocyte ratio ranging from 1:1 to 3:1 (median 2:1) (Figure 4). All MLEL as well as the BLEL in the HIV-positive patient showed a T-/B-lymphocyte ratio ranging from 1:2 to 1:6 (median 1:3) (Figure 5). Table 1 shows the clinical, pathological and EBV *in situ* hybridization findings of the 18 cases studied.

Discussion

Studies have shown that normal parotid tissue can show expression of the EBV.¹⁶ Viral expression has also been seen in BLEL.⁸ This expression has even been seen in cases of BLEL diagnosed as Sjögren's syndrome.²³ Most of these studies used DNA amplification techniques, namely, polymerase chain reaction (PCR), for the identification of viral expression. This suggests that the number of viral copies in these lesions is usually too low for detection by *in situ* hybridization. The number of benign LELs and cysts has been steadily rising with the AIDS epidemic.^{11,12} The lesions have been increasingly seen in HIV-positive patients.^{13,14} This may suggest an underlying immune mechanism for the development of these lesions.

Molecular studies have shown that a specific strain of EBV is seen in nasopharyngeal carcinoma and MLEL of the parotid gland.^{24,25} This strain is related to the Asian EBV type 1 virus. The strain shows tropism for epithelial rather than lymphoid cells. This probably explains the lack of expression of EBV mRNA in the lymphoid tissue in our MLELs. Viral mRNA expression in our BLEL of the HIV-positive patient was also seen exclusively in the epithelium and not the lymphoid tissue. This suggests that

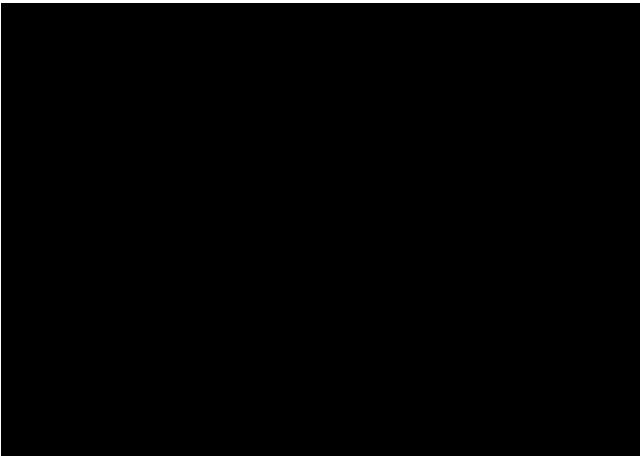


FIGURE 4A. BLLEL lesion showing a T-lymphocyte (A) to B-lymphocyte (B) ratio of approximately 1:1 (immunoperoxidase, 250x).

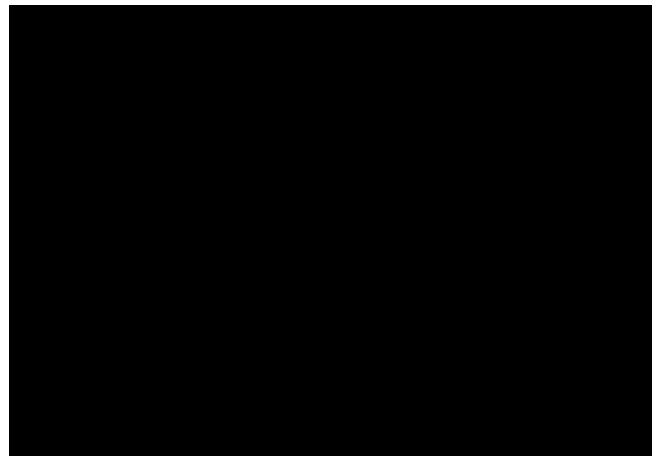


FIGURE 4B. BLLEL lesion showing a T-lymphocyte (A) to B-lymphocyte (B) ratio of approximately 1:1 (immunoperoxidase, 250x).



FIGURE 5A. MLEL lesion showing a T-lymphocyte (A) to B-lymphocyte (B) ratio of approximately 1:3 (immunoperoxidase, 250x).



FIGURE 5B. MLEL lesion showing a T-lymphocyte (A) to B-lymphocyte (B) ratio of approximately 1:3 (immunoperoxidase, 250x).

EBV present in both benign and malignant LEL could belong to the same viral strain. The epithelial cells have been shown to express a specific receptor that shows affinity for EBV. The receptor has been identified as the receptor for the c3d fragment of the complement.²⁶ Monoclonal antibodies to that receptor have been shown to react with parotid epithelium. To date, close to 100% of cases of MLELs expressing EBV have been seen in native Canadians. Cases of MLELs in patients of Western descent have not shown expression of the virus.²⁸ There was, however, viral expression in our case. Epidemiological data have shown that Canadian and United States natives are more prone to specific types of malignancy.²⁹ These types include MLEL.³ These native populations also show an increased incidence of infectious diseases with a relatively high morbidity and mortality from such diseases.^{30,31} These diseases include pulmonary tuberculosis with its associated immunosuppression.³² The above findings suggest that the American and Canadian

natives are more exposed to a specific strain of EBV, showing predilection for the epithelium of the parotid gland. These native populations may conceivably have a high incidence of immunosuppression due to the low socioeconomic conditions and high incidence of infectious diseases.

Studies of the immune system of patients with nasopharyngeal carcinoma have shown that there is a significant decrease in the number of circulating T-lymphocytes in patients with the neoplasm when compared to those patients in remission.^{33,34} Additionally, it has been shown that aggressive forms of nasopharyngeal carcinoma have a lower number of infiltrating lymphocytes.³⁴ Immunohistochemical evaluation of infiltrating lymphocytes in nasopharyngeal carcinoma has revealed a significant increase in the numbers of infiltrating B-lymphocytes when compared to T-lymphocytes.³⁵ MLELs of the parotid gland are closely related to nasopharyngeal carcinoma in terms of

morphology, epidemiology, pathogenesis in relation to EBV, and biological behavior. The advantage of MLEL as a tumor model over nasopharyngeal carcinoma is the existence of a benign counterpart for MLELs allowing the study of the natural history of the neoplasm in the parotid gland.

We therefore would like to propose a pathogenetic mechanism for carcinogenesis of MLELs. We believe that EBV may be present in the majority of BLELs. The level of expression may, however, be too low for detection by *in situ* hybridization. Viral replication in these benign lesions is probably inhibited by a competent immune system of T-lymphocytes preventing viral replication. When this immune system is disturbed, EBV replication becomes uninhibited, leading to higher nuclear levels of EBV expression, contributing to carcinogenesis. Our evidence for this immune-mediated hypothesis is based on the uniform expression of EBV in MLELs, the focal expression of the virus in the HIV-positive patient and the significant decrease in the number of infiltrating T-lymphocytes in both benign and malignant lesions expressing the virus. None of the lesions with predominance of infiltrating T-lymphocytes showed any viral expression. This pathogenetic pathway may conceivably apply to other lymphoepithelioma-like carcinomas of other organs, including nasopharyngeal carcinoma. Our hypothesis suggests a causal relationship between EBV and MLEL. Further investigations are therefore required to rule out or confirm this association, as the relationship between EBV and these lesions may be just incidental or circumstantial.

The study of the immune system of patients with benign and MLEL is warranted, as it may shed more light on the possible pathogenetic pathways of this malignant neoplasm. The findings may also provide some data pertinent to viral oncogenesis in general. More sensitive methods of viral identification such as PCR may prove beneficial in assessing the true incidence of EBV expression in benign LELs.

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