Malignant Lymphoma of the Thyroid and Epstein-Barr Virus

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Abstract

We have investigated the specific immune response to Epstein-Barr virus (EBV) of peripheral blood mononuclear cells (PBMC) from patients with malignant lymphoma of the thyroid. Coculture of PBMC and EBV resulted in EBV cell transformation and regression which was assayed by an EBV-induced B cell focus-regression assay technique. The EBV had been isolated from mouthwash samples. The specific immune response to EBV by outgrowth inhibition in PBMC from untreated EBV-seropositive patients with malignant lymphoma was significantly decreased when compared to PBMC from EBV-seropositive healthy subjects ($p < 0.05$). This observation is at least consistent with the possibility that B-cell proliferation after continuous or recurrent EBV infection could be a causative factor or may potentiate malignant lymphoma of the thyroid.

Malignant lymphoma of the thyroid occurs at the rate of 5% among thyroid neoplasms (Stauton *et al.*, 1973), and most prove to be cases of B-cell lymphoma (Maurer *et al.*, 1979; Aozasa *et al.*, 1986).

The ubiquitous Epstein-Barr virus (EBV), which can readily transform normal human B-cells *in vitro* to blast cells with infinite replicative ability, frequently leads to a self-

limiting lymphoproliferative disease, e. g., infectious mononucleosis (Henle and Henle, 1981). The EBV may also be implicated in the etiology of some cases of Burkitt's lymphoma (Old *et al.*, 1966) and nasopharyngeal carcinoma (Tamada *et al.*, 1984). Polyclonal and monoclonal B-cell lymphomas induced by EBV have occurred after infectious mononucleosis (Abo *et al.*, 1982) and renal transplantation (Hanto *et al.*, 1983). Moreover, the EBV antibody titers in patients with malignant lymphoma exceed those observed in patients with Hashimoto's thyroiditis (Matsubayashi *et al.*, 1987). Of course,

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whether this finding relates to pathogenetic factors or is secondary, is not clear.

In this study, we have analyzed the EBV-specific immune response in peripheral blood mononuclear cells (PBMC) from six patients with malignant lymphoma of the thyroid. The possible relationship of these findings to the etiology of malignant lymphoma of the thyroid is discussed below.

Materials and Methods

Blood samples and mouthwash samples were obtained from six patients with malignant lymphoma of the thyroid (2 men and 4 women; 47–68 years), who were all in stage I of the disease. Their EBV immune response was compared with data from seven healthy age-matched controls (2 men, 5 women; 48–72 years) who were EBV-seropositive donors. The diagnosis of malignant lymphoma was based on findings in the biopsy specimens classified by the morphologic criteria of Kiel (Lennert, 1981): three were centroblastic/centrocytic, one was immunoblastic, one was centrocytic, and one was lymphoplasmacytic/lymphoplasmacytoid. The expression of immunoglobulin (Ig) light chains in the proliferating cells was restricted in all of the patients. In none of the six patients or seven controls were adult T-cell leukemia virus associated antigens present in these sera; this was checked before the study. Sera and peripheral mononuclear cells (PBMC) were separated by the Ficoll technique. PBMC were resuspended in the medium (RPMI 1640 medium containing heated 10% fetal calf serum), adjusted to 1×10^6 cells/ml, and cultured at 37°C in a CO₂ incubator (5% CO₂) before EBV adhesion. Mouthwash samples were obtained after the subjects gargled for 30 seconds with 150 ml of RPMI 1640 medium.

EBV antibodies

The titers of IgG to the viral capsid antigen of EBV (EBVCA) and early antigen IgG (EBVEA) and of antibodies to EBV-determined nuclear antigen (EBVNA) were assayed in all serum samples before performing the test of EBV-specific cytotoxic T-cell function (see below). Anti-EBVCA and EBVEA titers were assayed

by indirect immunofluorescence, and anti-EBVNA titers by an anti-complement immunofluorescent method (Henle and Henle, 1978). Serial twofold dilutions starting at 1:10 were used to obtain the antibody titers. Titers of 1:10 or greater were considered to be positive for EBVCA, EBVEA, and EBVNA.

EBV-specific immune response

The EBV-specific immune response was investigated by using out growth inhibition (Moss *et al.*, 1978; Rickinson *et al.*, 1981; Moss *et al.*, 1981), also, as published previously by one of us (Katsuki, 1987); briefly, EBV (B95-8) was obtained by the culture of EBV-producing cells (3×10^5 cells/ml) with a viability of over 90% for 7–10 days at 37°C, the medium then being centrifuged at 2,000 rpm for 10 min. The supernatant was passed through a membrane filter with 0.45 μ m pores and kept at –80°C before co-culture of PBMC. PBMC (1.6×10^6) were incubated with 0.3 ml of EBV at 37°C (EBV adhesion to mononuclear cells). The cells were then washed twice in the medium. 2.5×10^4 cells, or cells concentrated twofold serially were seeded into six wells each in a 96-well culture dish and cultured at 37°C for 50 days. Each well contained 150 μ l of medium. Half of the medium was changed every 3–5 days. At the end of culture, the establishment of EBV-transformed cells or the regression of EBV-transformed cells in each well was investigated by microscopic observation; the appearance of progressively growing foci of transformed cells was detected in the establishment of EBV-transformed cells. The regression index (the initial cell concentration required to achieve 50% incidence of regression in EBV-infected cell cultures; IR₅₀/ml) was calculated by the method of Reed-Muench (Moss *et al.*, 1978); $\sqrt{a/3} \times b$ (a: number of wells showing transformation cells, b: initial seeded PBMC concentrations divided 10⁴).

Isolation of EBV from mouthwash samples

Transforming EBV were assayed with umbilical cord lymphocytes (Katsuki and Hinuma, 1975). Both 1×10^6 lymphocytes from umbilical cord blood and 1.0 ml mouthwash samples serially diluted tenfold, starting at 1:10, were incubated at 37°C for 1 hr. The virus first adhered to the lymphocytes; then the cells were washed and resuspended in medium and cultured at 37°C

Table 1. Titers of antibodies to EBV- and ATLV-associated antigens in sera

Donor	Age/Sex	IgG Antibody titer to			
		ATLA ¹	VCA ²	EA ³	EBNA ⁴
With malignant lymphoma of the thyroid					
1	47/F	<10	640	80	80
2	68/M	<10	160	<10	80
3	66/M	<10	5120	160	640
4	59/F	<10	640	1280	40
5	57/F	<10	40	<10	80
6	54/F	<10	160	<10	20
EBV-seropositive healthy controls					
1	63/F	<10	320	<10	40
2	48/M	<10	160	<10	40
3	58/F	<10	80	<10	40
4	72/F	<10	320	20	160
5	66/F	<10	160	<10	80
6	56/M	<10	40	<10	40
7	70/F	<10	160	20	80

¹ Adult T-cell leukemia viral antigen. ² Viral capsid antigen. ³ Early antigen (DR complex).

⁴ EBV-determined nuclear antigen. Titers of EBV-antigen antibodies in the two groups were similar.

for 50 days. Half the medium was changed every 3-5 days. Transformed B-cell lines (EBVNA positive) detected by an anti-complement immunofluorescent method (Katsuki and Hinuma, 1975) were judged to be positive for EBV. There were 5 wells for each dilution.

Statistical analysis

Wilcoxon's rank-sum test was used for statistical analysis.

Results

EBV antibodies

The results of tests for antibodies to EBVCA, EBVEA, and EBVNA are shown in Table 1. All six patients and seven controls had EBVCA and EBVNA antibodies in the serum. The titers of anti-EBVCA, EBVEA, and EBVNA in two groups were similar.

EBV-specific immune response

The incidence of transformation in the well culture of PBMC infected by EBV is

shown in Table 2. The regression index (mean $\pm$ SD) in the patients and in the controls was 35.4 ± 26.9 and 10.2 ± 3.7 , respectively, the difference being significant ($p < 0.05$).

Isolation of EBV from mouthwash samples

EBV-infected umbilical B-cells (transformed B-cells) were observed in three of the six patients and in one of the seven controls ($p < 0.1$, not significant) (Table 3).

Discussion

The association between malignant lymphoma of the thyroid and Hashimoto's thyroiditis may indicate a causal relationship in which chronic antigenic stimulation of lymphocytes causes their eventual transformation into lymphoma cells (Burke *et al.*, 1977, Holm *et al.*, 1985; Kato *et al.*, 1985). However, the factors that transform the

Table 2. Incidence of transformation in cultures of peripheral blood mononuclear cells infected with EBV (B95-8)

Donor	Number of culture well showing transformation cell concentrations						Regression	
	× 10 ⁴ /ml of peripheral mononuclear cells						index ¹	
	80	40	20	10	5	2.5		
With malignant lymphoma of the thyroid								
1	0/6	0/6	0/6	4/6	6/6	5/6	11.9	35.4 ± 26.9*
2	1/6	4/6	6/6	6/6	6/6	6/6	51.3	
3	0/6	2/6	5/6	6/6	5/6	3/6	31.3	
4	3/6	4/6	6/6	6/6	6/6	6/6	80.0	
5	0/6	0/6	0/6	1/6	5/6	6/6	7.1	
6	0/6	2/6	5/6	6/6	6/6	6/6	31.3	
EBV-seropositive healthy controls								
1	0/6	0/6	0/6	6/6	6/6	6/6	14.1	10.2 ± 3.7
2	0/6	0/6	0/6	0/6	5/6	6/6	6.6	
3	0/6	0/6	0/6	1/6	6/6	6/6	7.6	
4	0/6	0/6	0/6	4/6	6/6	6/6	11.9	
5	0/6	0/6	0/6	0/6	3/6	6/6	5.0	
6	0/6	0/6	1/6	4/6	6/6	6/6	12.8	
7	0/6	0/6	0/6	5/6	6/6	6/6	13.2	

¹ Initial cell concentration required to achieve 50% incidence of regression in EBV-infected cell cultures.

* $p < 0.05$ vs. regression index in controls. Results are expressed as means $\pm$ SD.

Table 3. Isolation of EBV from mouthwash samples

Donor	Dilution of mouthwash samples			
	1	10 ⁻¹	10 ⁻²	10 ⁻³
With malignant lymphoma of the thyroid				
1	5/5	5/5	2/5	0/5
2	4/5	1/5	0/5	0/5
3	0/5	0/5	0/5	0/5
4	5/5	2/5	0/5	0/5
5	0/5	0/5	0/5	0/5
6	0/5	0/5	0/5	0/5
EBV-seropositive healthy controls				
1	0/5	0/5	0/5	0/5
2	0/5	0/5	0/5	0/5
3	5/5	1/5	0/5	0/5
4	0/5	0/5	0/5	0/5
5	0/5	0/5	0/5	0/5
6	0/5	0/5	0/5	0/5
7	0/5	0/5	0/5	0/5

$p < 0.1$ for malignant lymphoma of the thyroid vs. EBV-seropositive healthy controls

reactive intrathyroidal lymphocytes to lymphoma cells are unknown.

In susceptible persons, EBV infects B-cells, followed by extensive proliferation of suppressor/cytotoxic T-cells (De Waele *et al.*, 1981); such cells help control the transformation of infected B-cells (Rickinson *et al.*, 1981). Abo *et al.* (1982) have suggested that monoclonal malignant lymphoma arises after infectious mononucleosis as a result of EBV infection. It is well-known that clonal B-cell expression and malignant lymphomas consisting of EBV-infected cells occur in immunosuppressed patients (Penn, 1986). On the other hand, EBV infection has been thought to be a causative factor in a subtype of both malignant non-Hodgkin's lymphoma (Wultzler *et al.*, 1986) and Hodgkin's lymphoma (Weiss *et al.*, 1987, Staal *et al.*, 1989) as suggested by Southern blotting analysis of EBV-DNA. By analogy, therefore, it may be considered that EBV

could be a possible causative factor in malignant lymphoma even in the absence of an immunoinufficient state. Patients with malignant lymphoma of the thyroid have higher titers of EBVNA antibodies than patients with Hashimoto's thyroiditis (Matsubayashi *et al.*, 1987), whereas patients with Hashimoto's thyroiditis frequently have higher titers of EBVNA than normal persons (Matsubayashi *et al.*, 1987). These findings have suggested that EBV may be important in the development of malignant lymphoma of the thyroid, either as a pathogenetic factor or as a consequence of the disease. In this study, we found a significant decrease in the EBV-specific immune response due to outgrowth inhibition (i. e., a long-term T-cell mediated immune reaction of PBMC to EBV measured by an EBV-induced B-cell focus-regression assay), as well as a slight tendency for EBV to be found more frequently in mouthwash samples in patients with malignant lymphoma of the thyroid than in EBV-seropositive healthy subjects. It is not yet evident whether these observations relate to the cause of the malignant lymphoma of the thyroid or are a consequence of it. If the former, it is possible that the impaired suppressor/cytotoxic T-cell response to EBV in the PBMC from patients with malignant lymphoma of the thyroid may allow EBV to readily proliferate. Conversely, if these effects are a consequence of the disease, then the decrease in EBV specific suppressor/cytotoxic function may reflect a vicious circle resulting in proliferation of B-cells in this disease. EBV is a common virus and could be present in lymphoreticular tissue (such as lymph nodes, tonsils and Hashimoto's thyroiditis), through the oral cavity and might transform B-cells in the neck region. To suggest a direct role of EBV infection in the development of malignant lymphoma of the thyroid, it would be necessary to find proviral DNA in the nuclei of the tumor cells of such

lymphomas.

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