T-Lymphocyte Activation Is Correlated With the Presence of Anti-EBV in Patients With Laryngeal Squamous Cell Carcinoma

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Abstract. Background/Aim: Chronic viral infection is an important risk factor in the development of cancer. Failure of immune response to clear the oncogenic infection can facilitate cancer progression. The aim of the present study was to analyze early and late activation of T-lymphocytes related to Epstein-Barr virus (EBV) infection by the expression of markers of activation (CD69, CD25) on the surface of T-lymphocytes (CD3+, CD4+, CD8+) in patients bearing laryngeal cancer according to absence/presence immunoglobulin G antibodies to EBV nuclear antigen (EBNA1). Materials and Methods: Thirty-three patients with laryngeal squamous cell carcinoma (LC) and 20 volunteers without cancer (control group) were enrolled in the study. Peripheral blood samples were collected from every individual. The markers of activation of T-lymphocytes were determined by flow cytometry, whereas commercial immunoenzymatic assay kits were used for detection of anti-viral capsid antigen (VCA) IgM, anti-VCA IgG, and anti-EBNA1 IgG. Results: Increased early activation of CD8+ and CD4+ T-lymphocytes was found in patients with LC. There was a significantly higher proportion of CD4+ and CD8+ T-lymphocytes expressing CD69 antigen in patients with LC compared to the control group. The proportion of CD4+ CD25+ T-lymphocytes in patients with LC positive for anti-EBNA1 IgG and anti-VCA IgM was lower compared to patients without antibodies to VCA IgM. Conclusion: The dysfunction of immune response in larynx cancer patients could be associated with EBV infection.

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present study, we analyzed early and late activation of T-lymphocytes in patients with EBV antibodies: immunoglobulin M antibodies and immunoglobulin G antibodies to the viral capsid antigen (anti-VCA IgM and anti-VCA IgG, respectively) and immunoglobulin G antibodies to EBV nuclear antigen (anti-EBNA1 IgG).

**Patients and Methods**

*Study participants.* Thirty-three previously non-treated patients with laryngeal squamous cell carcinoma (LC group; aged between 40 and 79 years) and 20 volunteers (control group; aged between 44 and 67 years) without cancer were enrolled in this study at the Department of Otolaryngology and Laryngeal Oncology of the Medical University of Lublin. The patients’ characteristics according to TNM staging system (15) are described in detail in Table I. The study was approved by the Local Medical Ethical Committee (KE-0254/70/2015).

Flow-cytometric analysis of T-lymphocytes and the expression of activation markers (CD69+ and CD25+). Peripheral blood samples were collected into EDTA-containing tubes and subjected immediately to cellular staining with the following mouse anti-human fluorescently labelled antibodies: fluorescein isothiocyanate (FITC)-conjugated CD3, FITC-anti-CD4, phycoerythrin (PE)-conjugated anti-CD8, phycoerythrin-cy5 (PE-cy5) anti-CD69, PE-cy5-anti-CD25, Pe-Cy5-anti-CD3. Appropriate isotype antibodies controls were used for each sample. All the antibodies and isotype controls were purchased from Becton Dickinson, Franklin Lakes, NJ, USA. The cells were stained for 30 min at 4°C in the dark. Then cells were treated with Lysing Solution (Becton Dickinson) for 10 min at 4°C in the dark. After staining, the cells were washed twice with phosphate-buffered saline. Stained cells were then subjected to flow-cytometric analysis using FACSCalibur flow cytometer (Becton Dickinson).

**Evaluation of anti-VCA IgM, anti-VCA IgG, anti-EBNA1 IgG concentration.** A plasma sample was taken from every patient and volunteer and stored at −80°C. Detection of anti-VCA IgM, anti-VCA IgG, anti-EBNA1 IgG was performed by means of commercial immunoenzymatic assay kits (all with a limit of detection of 10 U/ml; Demeditec, Kiel, Germany) according to the manufacturer’s instructions.

**Statistical analysis.** The experimental data were analyzed by means of Mann–Whitney *U*-test using Statistica 12 software (StatSoft, Tulsa, OK, USA). Differences between the groups at *p*<0.05 was considered statistically significant.

**Results**

All patients in the LC group were anti-VCA IgG-positive and, regardless of TNM status, were divided into two main groups according to anti-EBNA1 IgG status. The EBNA1 IgG-positive group was subsequently divided into two additional groups according to whether they were anti-VCA IgM-positive or -negative. The volunteers in the control group were all anti-VCA IgG and anti-EBNA1 IgG-positive.

**Early response of CD4+ T-helper lymphocytes to antigens, as shown by CD69 expression, is increased regardless of EBNA1 status in patients with LC.** Abnormal lymphocyte turnover with a decrease of CD4+ T-lymphocytes is a characteristic feature in patients with cancer. An increased proportion of CD4+ T-lymphocytes is a sign of significantly improved prognosis for patients with cancer (16). In our study, we observed significantly lower proportions of CD3+ T-lymphocytes (*p*<0.001) and CD4+CD3+ T-lymphocytes (*p*<0.002) in anti-EBNA1 IgG-positive patients compared with the control group. In contrast, in anti-EBNA1 IgG-negative patients, the proportion of CD3+ T-lymphocytes compared to the control group did not statistically significantly differ. Similarly to the anti-EBNA1-positive group of patients, the proportion of CD4+CD3+ T-lymphocytes in the anti-EBNA1-negative LC group was significantly lower compared with controls (*p*=0.033). Our results demonstrate that the decline of T-helper population is independent of anti-EBNA1 IgG status and is characteristic of patients with LC (Table II).

We observed that the proportions of CD3+CD69+ (*p*=0.038) and CD4+CD69+ (*p*=0.026) T-lymphocytes in anti-EBNA1 IgG-positive patients were statistically higher than those in controls (*p*=0.038). Similarly, in anti-EBNA1 IgG-negative patients, the proportion of CD4+CD69+ T-lymphocytes was significantly higher in comparison with the control group (*p*=0.007), showing intense immune response to tumor antigens (Table III).

**Both early and late cytotoxic CD8+ T-lymphocyte responses were significantly increased in patients with LC, regardless of anti-EBNA1 IgG status.** In the present study, the differences in the proportion of CD8+CD3+ lymphocytes among patients with LC and the control group were not significant, regardless of anti-EBNA1 IgG status (Table II). However, we showed that the proportion of CD8+CD69+ T-lymphocytes in anti-EBNA1 IgG-positive patients with LC was significantly higher than in controls (*p*<0.001). Similarly, the proportion of CD8+CD25+ T-lymphocytes was significantly higher in both anti-EBNA1 IgG-positive (*p*<0.001) and -negative patients with LC (*p*=0.004) compared with the controls (Table III), thereby our results suggest early and late CD8+ T-lymphocyte activation.

**Decrease of late response of CD4+CD25+ T-lymphocytes may be associated with the presence of antibodies to VCA IgM in anti-EBNA1 IgG-positive patients with LC.** CD4+CD25+ T-cells consist of activating and T-regulatory cells (Tregs); Tregs express higher levels of CD25 than do activated T-lymphocytes (17). Our results showed a significantly higher proportion of CD4+CD25+ T-lymphocytes in anti-EBNA1 IgG-positive patients with compared with the control group (*p*=0.027). In contrast, no significant differences between the proportion of
CD4\(^+\)CD25\(^+\) T-lymphocytes in anti-EBNA1 IgG-negative patients and the controls were found, meaning that in anti-EBNA1 IgG-negative patients, dysfunction of immune response was observed. We also suggest involvement of regulatory response mediated by CD4\(^+\) T-cells with high expression of CD25 antigen in anti-EBNA-IgG-positive patients (Table III). The presence of anti-EBV antibodies (anti-VCA IgM, anti-VCA IgG, anti-EBNA1 IgG) in patients indicates recent or recurrent infection (18). Interestingly, the proportion of CD4\(^+\)CD25\(^+\) T-lymphocytes in anti-EBNA1 IgG/anti-VCA IgM-positive patients with LC was lower compared to patients without antibodies to VCA IgM: 48.55% (29.61%-74.61%) versus 67.69% (33.09%-80.27%), respectively (p=0.040), supporting our hypothesis that lower expression of late markers during recent infection or recurrent phase of EBV infection may be associated with dysfunction of immune response.

**Discussion**

CD4\(^+\) T-lymphocytes play a pivotal role in maintaining anticancer immune response (19). An increased proportion of these cells in the tumor environment was correlated with more favorable prognosis for patients with head and neck cancer (16). It was also shown that EBV infection is associated with a reduction of the number of CD4\(^+\) T-lymphocytes in patients with EBV-related cancer (20). Despite the involvement of EBV infection in the development of cancer having been extensively studied (5, 8, 9, 21-27), reports analyzing the initial phase of immune response and lymphocyte activation affected by viral infection are rare. During primary EBV infection, antibodies to VCA IgM are generated and persist from weeks to months. Antibodies to EBNA1 IgG appear later and suggest an ongoing infection. In contrast, infection-induced antibodies to VCA IgG show a lifelong persistence, with different amounts fluctuating over time after EBV infection (28). EBV infection subsequently inhibits the activation of EBV-specific CD4\(^+\) lymphocytes (29). Thus, the effector cell response does not eliminate EBV infection, which results in latent infection (30).

Lymphocyte imbalance with a decrease in CD4\(^+\) T-lymphocytes was observed in patients with LC (31). Similarly, our results showed significant decrease of CD4\(^+\) T-lymphocytes in both anti-EBNA1-positive and -negative patients with LC. Additionally, no differences were observed in CD8\(^+\) T-lymphocytes in these groups. Thereby, our data indicate that the presence of antibodies to EBNA1 IgG is not associated with reduction of CD4\(^+\) T-lymphocytes.
The activation of T-lymphocytes is an important immunological process in the recognition of tumor antigens. Our data indicate increased early activation of CD8+ and CD4+ T-lymphocytes in patients with LC compared to the control group. Similarly to another report, showing higher expression of CD69 on T-lymphocytes in patients with LC (13), we demonstrated the appropriate increase of molecules of early and late activation of CD8+ T-lymphocytes (CD69 and CD25), whereas the number of CD8+ T-lymphocytes was not changed. The reduction of CD4+ T-lymphocytes and abnormal CD8+ T-lymphocyte activation were showed in patients with EBV-associated hemophagocytic lymphohistiocytosis patients (32). Lymphocyte activation predicts survival in patients with head and neck cancer. It was suggested that the degree of lymphocyte activation may reflect tumor-infiltrating T-lymphocyte function. The high expression of CD69 was found to impair the prognosis of cancer (33). We tried to see whether the proportion of lymphocytes with CD69 expression was impaired with reduction of the CD25+ T-lymphocyte population might be associated with the increase of Tregs. Tregs are suspected to contribute to the promotion of viral persistence by inducing immunosuppressive factors (interleukin-10, transforming growth factor) (34), and local accumulation of Tregs facilitates tumor development (35). One study suggested that Tregs can prevent the EBV-specific T-cell response in order to control immunopathological damage manifesting as infectious mononucleosis. The development of symptoms of primary EBV infection (infectious mononucleosis) results in the activation of CD8+ T-lymphocytes due to the diminishing of Tregs (34). We observed no differences between the proportion of CD4+CD25+ T-lymphocytes in anti-EBNA1 IgG-negative patients with LC compared to the control group, suggesting dysfunction of immune response. Interestingly, the proportion of CD4+CD25+ T-lymphocytes was significantly lower in anti-EBNA1 IgG-positive patients with EBV antigens than in those without the latter antibodies. Similar results were reported, showing a decreased number of T-lymphocytes with CD25 expression in patients bearing LC (13). However, we showed that differences in the proportion of CD4+CD25+ T-lymphocytes may be associated with exposure to EBV antigens. Our data indicate an increased early activation of T-lymphocytes with reduction of the CD25+ population during recent or recurrent of EBV

Table II. Subsets of lymphocytes (%) in patients with laryngeal cancer (LC) and the control group.

| Subsets of lymphocytes | LC (n=33) | EBNA1 IgG-positive (n=24) | p-Value* | EBNA1 IgG-negative (n=9) | p-Value* |
|------------------------|-----------|--------------------------|----------|--------------------------|----------|
| CD3+                   | 76.49 (63.34-83.08) | 68.26 (51.43-84.20) | <0.001   | 71.31 (45.92-81.68) | 0.285    |
| CD4+CD3+               | 50.02 (36.67-62.12) | 39.20 (18.40-60.53) | 0.002    | 41.04 (32.10-55.94) | 0.033    |
| CD8+CD3+               | 23.79 (12.88-47.66) | 24.73 (10.05-42.00) | 0.877    | 25.69 (10.46-36.13) | 0.789    |

EBNA1: Epstein–Barr nuclear antigen. Data are median (range). *Versus the control group.

Table III. Percentage of lymphocytes expressing CD69 and CD25 antigens in patients with laryngeal cancer (LC) and the control group.

| Antigens expressed | LC (n=33) | Control group (n=20) | EBNA1 IgG-positive (n=24) | p-Value* | EBNA1 IgG-negative (n=9) | p-Value* |
|--------------------|-----------|----------------------|--------------------------|----------|--------------------------|----------|
| CD3+CD69+          | 3.13 (1.77-6.39) | 4.07 (1.40-11.14) | 0.038                     | 8.90 (1.72-12.32) | 0.182 |
| CD4+CD69+          | 2.91 (1.5-7.58)  | 4.23 (0.81-13.84) | 0.026                     | 8.14 (2.00-10.11) | 0.007 |
| CD8+CD69+          | 0.80 (0.29-1.27) | 3.68 (1.09-8.85) | <0.001                    | 3.7 (1.06-10.99) | <0.001 |
| CD3+CD25+          | 30.13 (26.05-58.34) | 39.83 (9.49-61.57) | 0.332                     | 42.25 (11.16-89.93) | 0.285 |
| CD4+CD25+          | 45.04 (39.82-67.74) | 59.43 (29.61-80.27) | 0.027                     | 61.60 (41.79-96.69) | 0.142 |
| CD8+CD25+          | 1.82 (0.55-3.90)  | 5.80 (0.94-80.41) | <0.001                    | 7.02 (1.68-85.94) | 0.004 |

EBNA1: Epstein–Barr nuclear antigen. Data are median (range). *Versus the control group.
infection and suggest that dysfunction of immune response in patients with LC might be associated with EBV infection.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors’ Contributions

Methodology, AH, EG; ASŁ; formal analysis: AH, EG, ASŁ, MT; laboratory tests: AH, ASŁ, BK, MT; patient qualification: JK, ASŁ; writing – original draft preparation: JK, AH, AS; funding acquisition: JK.

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