PRESENCE OF HERPESVIRUSE DNA IN LYMPHOCYTES ISOLATED FROM PATIENTS WITH BLOOD DISORDERS

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Abstract

The aim of this study was to check the presence of DNA of five human herpesviruses in lymphocytes isolated from peripheral blood of people with lymphoproliferative diseases and to investigate the relationship between these disorders and the presence of the viruses. A total of 113 blood specimens from patients with different blood disorders and 96 specimens from healthy people (control group) were obtained. Among 113 specimens from patients with blood disorders, DNA of EBV in 39.8%, CMV in 30.1%, HHV6 in 8.0%, HHV7 in 20.4%, and HHV8 in 9.7% of the people was detected. Among 96 specimens from healthy people, DNA of EBV was detected in 9.3%, CMV in 27.1%, HHV6 in 4.2%, HHV7 in 13.5%, and HHV8 in 4.2%. It was found that the differences in presence and number of viruses between groups were very significant. Significantly, a larger detection of DNA of EBV in blood disorder group was observed. These results suggest the participation of EBV in blood disorders.

Key words: human population, EBV, HHV8, blood disorders, lymphocytes.

Epstein-Barr virus (EBV) infects more than 90% of the human population but the infection can have a wide range of health consequences (25). EBV enters via the oropharyngeal route, infects resting B-cells and/or epithelial cells (18). Primary infection usually occurs during childhood without any symptom or as a mild illness. During adolescence, infectious mononucleosis characterised by fever, lymphadenopathy, and pharyngitis occur in around 50% of individuals (6). This most extensively studied γ-herpes virus is also associated with various human malignancies such as Hodgkin’s and non-Hodgkin’s lymphomas, Burkitt lymphoma, nasopharyngeal carcinoma, EBV-gastric carcinoma, AIDS-related lymphoproliferative disorders, and X-linked lymphoproliferative disorder (Duncan’s disease). All these tumours are associated with the EBV latency cycle (1, 11, 13). EBV can transform and immortalise resting B-cells in cultures, which suggests that it could have oncogenic specificity. EBV has a wide spectrum of proteins, which mimic cellular proteins regulating cell cycle. These proteins interact with or exhibit homology to a wide variety of anti-apoptotic molecules, cytokines, and signal transducers, hence promoting EBV infection, cell immortalisation and transformation. For example, BHRF1, viral homologue of cellular Bcl-2 protein, can block apoptosis by the inhibition of pro-apoptotic BAX and BAK protein. The second EBV vBcl-2 homologue, BALF1, may either block apoptosis or antagonise BHRF1. BCRF1 protein shows 84% sequence homology to human IL-10, which inhibits the activation and function of T-cells, monocytes, and macrophages, and is the activation factor for B-cells. Another viral protein, BDLF2, is a homologue of human cyclin B1, which regulates the G2-M transition in the cell division cycle by activating protein kinases (7, 28, 29). EBV latent membrane protein (LMP-1) interferes with intracellular TNF receptor-associated factors (TRAFs) that can activate transcription factors, like nuclear factors κB and jun-N-kinase. Activation of NFκB leads to the transcription of over 100 genes, like cytokines, adhesion molecules, chemokines, and others (15, 27, 31). Oncogenic properties of LMP-1 were confirmed by in vivo and in vitro experiments (14).

Over expression of Bcl-2 and over expression or activation of NFκB was detected in numerous cancers. In haematopoetic cancers, over expression of NFκB was detected in 50% of cancer cases coming from B-lymphocytes (NHL and B-CCL) (17, 24).

BARTF-1 produces a protein that shows homology to the intracellular adhesion molecule 1 as well as the human colony stimulating factor 1 receptor. BARTF-1 oncogene is expressed in nasopharyngeal carcinoma (NPC) and gastric (GC) carcinoma (10).

Human herpesvirus 8 (HHV8) has been identified in skin lesions of patients with Kaposi’s sarcoma and has also been detected in primary effusion lymphoma and in multicentric Castleman’s disease (28).
HHV8 vcr1-2 inhibits apoptosis induced by BAX (3, 4, 5). Recent experiments and clinical observations support the possible involvement of other lymphotropic herpesviruses (human herpesviruses 6 and 7) in human lymphoproliferative diseases (20).

The aim of this study was to analyse for the presence of the DNA of five human herpesviruses in lymphocytes isolated from peripheral blood of people with lymphoproliferative diseases and to investigate the relationship between these disorders and the presence of the viruses.

Material and Methods

Specimens. Peripheral blood specimens from 113 patients (55 women, 58 men, aged from 21 to 82 years) with different blood disorders (44 patients with leukaemias, 32 with lymphomas, 17 with myelomas, and 20 with other disorders) and 96 specimens from healthy people (control group) were obtained. The specimens were received from the Warsaw Institute of Haematology and Transfusiology.

Isolation of leukocytes. Leukocytes were isolated from whole blood using LSM® Lymphocyte Separation Medium (ICN Biomedicals Inc.) according to the instruction.

Isolation of DNA. Total DNA was isolated from the leukocytes using QIAamp® DNA Mini Kit (QIAGEN) according to the instruction. A nested-polymerase chain reaction to detect a highly conserved region within DNA polymerase gene was performed.

Nested-PCR assay. A 5 µl aliquot of DNA sample was amplified in a reaction mixture (total volume of 50 µl) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM MgCl₂, 200 µM each dNTP, 10 pmol of forward and reverse primer for appropriate herpesvirus and 1.25 U of Taq polymerase (Perkin-Elmer). An initial denaturation step at 94°C for 2 min was followed by 30 cycles, consisting of 30 s at 94°C, 1 min at 53°C, and 30 s at 72°C. A final extension step at 72°C was carried out for 5 min. After the first amplification round, 1 µl of the reaction product was added to 49 µl of the second round reaction mixture consisting of 60 mM Tris-HCl (pH 8.5), 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 200 µM each dNTP, 10 pmol of forward and reverse primer for appropriate herpesvirus and 1.25 U of Taq polymerase (Perkin-Elmer). An initial denaturation step at 94°C for 2 min was followed by 30 cycles, consisting of 30 s at 94°C, 1 min at 47°C, and 30 s at 72°C. A final extension step at 72°C was carried out for 5 min. Ten microlitres of the second round amplification product was analysed by electrophoresis in 4% agarose (Sigma) 1x TBE gel stained with ethidium bromide (0.5 µg/mL) (22).

Forward primers were designed to render the amplification products of different size: for HHV6 (68 bp), HHV7 (122 bp), HHV8 (97 bp), CMV 78 bp), and EBV (54 bp). Primer pairs specific for each herpesvirus are listed in Table 1 (22).

Statistical analysis. The obtained results were analysed by the Fisher exact test.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ -3’</th>
</tr>
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<tbody>
<tr>
<td>HHV6/1+</td>
<td>GAG GTG AYA TTY TAT GGT GAT ACG GA</td>
</tr>
<tr>
<td>HHV6/1-</td>
<td>TGT CTA CCA ATR TAT CTT TTT TT</td>
</tr>
<tr>
<td>HHV6/2+</td>
<td>GCC AAA CAT ATC ACA GAT CG</td>
</tr>
<tr>
<td>HHV6/2-</td>
<td>GGA CAT AAA ATC TTV TCR AAC TC</td>
</tr>
<tr>
<td>HHV7/1+</td>
<td>GAG GTG AYT TAT GGT GAT ACT GA</td>
</tr>
<tr>
<td>HHV7/1-</td>
<td>AAC TTT CCA ATG FAA CGT TTC TT</td>
</tr>
<tr>
<td>HHV7/2+</td>
<td>GTT ACT TTC AAA AAT GTT TGT CCC</td>
</tr>
<tr>
<td>HHV7/2-</td>
<td>GGA AAT AGG ATC TTT TCA AAT TC</td>
</tr>
<tr>
<td>HHV8/1+</td>
<td>AAG GTC ATA TAC GGC GAC ACT GA</td>
</tr>
<tr>
<td>HHV8/1-</td>
<td>AGT ACC CCC ACV TAT CTC TTC TT</td>
</tr>
<tr>
<td>HHV8/2+</td>
<td>GGA CAG CGT GTC AGA CTT CG</td>
</tr>
<tr>
<td>HHV8/2-</td>
<td>CTI GAA GAT CTT TTC AGC CTC</td>
</tr>
<tr>
<td>CMV/1+</td>
<td>CGG GTC ATC TAC GGG GAC AGC GA</td>
</tr>
<tr>
<td>CMV/1-</td>
<td>ACT TGT CCG ATG TAA CTT TTI TT</td>
</tr>
<tr>
<td>CMV/2+</td>
<td>GGG CCC AGC CTG GCG CAC TA</td>
</tr>
<tr>
<td>CMV/2-</td>
<td>GAC GAA GAC CTT TTC AAA CTC</td>
</tr>
<tr>
<td>EBV/1+</td>
<td>CGA GTC ATC TAC GGG GAC AGC GA</td>
</tr>
<tr>
<td>EBV/1-</td>
<td>AGG ACC CCC ACA TAT CTC TTC TT</td>
</tr>
<tr>
<td>EBV/2+</td>
<td>ACC CGG AGC CTG TTT GTA GC</td>
</tr>
<tr>
<td>EBV/2-</td>
<td>GGA GAA GGT CTT CTC GGC CTC</td>
</tr>
</tbody>
</table>

Results

Among 113 specimens from patients with blood disorders, DNA of EBV was obtained in 45 (39.8%), CMV in 34 (30.1%), HHV6 in 9 (8.0%), HHV7 in 23 (20.4%), and HHV8 in 11 (9.7%).

Among 96 specimens from healthy people, DNA of EBV was detected in 9 (9.3%), CMV in 26 (27.1%), HHV6 in 4 (4.2%), HHV7 in 13 (13.5%), and HHV8 in 4 (4.2%) (Fig. 1).

The differences in the presence and number of viruses between groups of patients with blood disorders and healthy people were found: no virus was detected in 43 (38.1%) people from the patient group and in 54 (56.3%) people from the control group. One virus was detected in 31 (27.4%) specimens from the group with blood disorders and in 31 (32.3%) specimens from the control group. Two viruses were found in 28 (21.5%) and in nine (9.4%), and three viruses in nine (8.0%) and in two (2.1%) specimens, respectively. Four viruses were found only in two (1.8%) specimens from patients with blood disorders (Fig. 2).

In the group of 44 specimens diagnosed as lymphomas, CMV was detected in 32 (34.4%), EBV in 13 (40.6%), HHV6 in 12 (28.1%), and HHV8 in two (4.5%) cases. In the group of 44 patients with leukaemias, CMV was detected in 11 (25.0%), EBV in 18 (40.9%), HHV6 in one (2.3%), HHV7 in seven (15.9%), and HHV8 in five (11.4%) cases. In the group of 17 myelomas, CMV was detected in three (17.7%), EBV in six (35.3%), HHV6 in two (11.8), HHV7 in three (17.7%), and HHV8 in two (11.8%) cases. In the group of different blood disorders...
CMV was detected in nine (45%), EBV in eight (40%), in two (10%) cases (Fig.3). HHV6 in two (10%), HHV7 in four (20%), and HHV8

Fig. 1. Detection of herpesviruses in lymphocytes isolated from patients and healthy people.

Fig. 2. Presence of viruses in lymphocytes from patients and healthy people.

Fig. 3. Detection of herpesviruses in lymphocytes isolated from peripheral blood from patient with blood disorders: leukaemias, lymphomas, myelomas, and others.
It was found out that differences in the presence and number of viruses between groups were very significant. Numbers of the detected herpesviruses were significantly higher in blood of the affected group. Association between rows and columns is very significant and the number and presence of viruses are essential.

A significantly higher detection rate of DNA of EBV in blood of the affected group was observed in general, as well as between patients with leukaemia, lymphoma, and myeloma and healthy ones. No differences were observed in the presence of DNA of EBV among blood samples of the affected group. Differences in the presence of DNA of other herpesviruses between patients with blood disorders and healthy ones were not significant.

**Discussion**

In recent decades, the participation of viruses in oncogenesis has been confirmed. Oncogenic viruses have the ability to immortalise infected cells and as a result cause uncontrolled proliferation and tumour transformation.

EBV, a causative agent of numerous human cancers uses its viral proteins, the action of which mimics several growth factors, transcription factors, and anti-apoptotic factors, to gain control over cellular pathways that regulate diverse homeostatic cellular functions.

Both γ-herpesviruses investigated in our study; EBV, and HHV8, have the capability to affect cellular pathways and have a potential oncogenic effect in the investigated blood disorders. However, the frequency of EBV DNA detection was higher than that of HHV 8 DNA. This increased frequency is not an effect of blood transfusion, because cytomegalovirus, other herpesvirus that could be transferred with blood, is detected on the same level in specimens from patients with blood disorders (30.1%) and from healthy people (27.1%).

In order to accept a virus as a tumourgenesis agent, an epidemiological relationship between the occurrence of the cancer and virus has to be found. The detection of EBV in tumour tissues in numerous studies was a method to confirm or deny the participation of the virus in the aetiopathogenesis of this disease. In recent years, the relationship between EBV and gastric cancer was confirmed. Ferrazzo et al. (9) studied the presence of EBV and HHV-8 DNA in HIV-related oral plasmablastic lymphoma. EBV was detected in seven cases and all cases were negative for HHV-8. They concluded that only EBV could play a role in the aetiopathogenesis of HIV-related oral plasmablastic lymphoma. EBV has been found in most cases of gastric lymphoepithelioma-like cancer and in small but significant proportion of gastric adenocarcinomas (12, 32). In 1993, Oda et al. (19) examined 22 cases of gastric carcinoma by PCR and in situ hybridisation. EBV DNA was detected in 14 (77.8%) cases. In another study, Lou et al. (16) checked 172 gastric carcinoma tissues and 172 corresponding para-carcinoma tissues and EBV DNA was found in 11 (6.39%) cancer samples and no EBV DNA was found in para-carcinoma tissues. Alves et al. (2) detected DNA EBV in 20 of 21 penile tumour samples using PCR reaction; they suggest that EBV can be implicated in the rise and/or progression of penile tumours. In our study, EBV was found in almost 40% of the specimens: in lymphomas group in 34.4%, in leukaemias in 40.9%, and in myelomas in 35.3%. In comparison, in the healthy people group, EBV was detected only in 9.3%. In specimens from patients with blood disorders, DNA of EBV was detected four times more often than in specimens from healthy people. Other herpesvirus, CMV, also was detected in high level in 30.1% of patients, but it was present also in 27.1% of healthy people, so there is no significant difference between these groups. Other herpesviruses were detected in low levels and there were not significant differences between patients and the control group. This result suggests that the blood disorders such as lymphoma, leukaemia, and myeloma can be related to the presence of EBV.

The following differences in the presence of herpesviral DNA were observed. No virus was detected in 56.3% of the healthy people group and only in 38.1% of patients with blood disorders. Four viruses were found only in the patients group. These differences are statistically significant and suggest that herpesvirus co-infection can be related to blood disorders.

The controversy regarding the association between the presence of EBV and breast cancer has recently been reported in two articles. Perrigoue et al. (21) measured the number of viral DNA molecules per cell in 45 tumour biopsies using real-time quantitative PCR. In no case was EBV DNA detected with either of two different probes at the level above 0.1 molecule per cell in two sections of tumour samples. The conclusion was that EBV did not contribute to progress of breast cancer. Deshpande et al. (8) studied the expression of EBV gene products: EBERs, EBNA1, LMP1, and LMP2 in breast cancer cells. They failed to detect the expression of any of the above-mentioned EBV gene products.

To confirm the relation between EBV infection and lymphoproliferative diseases, we are planning in the future to carry out tests on the expression of EBV proteins, which change cell cycle.

**References**