REACTIVATION AND MULTIPLICATION OF LYMPHOTROPIC HUMAN HERPESVIRUSES (HHV-4, HHV-5, HHV-6 ÉS HHV-8) IN PATIENTS SUFFERING FROM PLASMA CELL DYSCRASIAS AND EOSINOPHILIC GRANULOMA

Ph.D. Thesis

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2006
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Budapest
2006
INTRODUCTION

The name for **herpesviruses** comes from the Antiquity and originates from the Greek word herpes, herpetos (meaning reptant or crawler, snake) that used to refer to the characteristic that it spreads along the nerves (herpes-zoster) of a skin deformation caused by one of the human herpesviruses. The members of the virus families containing more than 130 different herpesviruses have already been isolated from all higher animal species representing the higher steps of evolution. According to our knowledge, eight herpesviruses are classified as human herpesviruses: Herpes Simplex Virus (HSV-1, Human Herpesvirus 1=HHV-1), Herpes Simplex Virus 2 (HSV-2, Human Herpesvirus 2=HHV-2), Varicella-zoster Virus (VZV, Human Herpesvirus 3 = HHV-3), Epstein-Barr Virus (EBV, Human Herpesvirus 4 = HHV-4), Cytomegalovirus (CMV, Human Herpesvirus 5 (HHV-5), Human Herpesvirus 6 (HHV-6A and HHV-6B), Human Herpesvirus 7 (HHV-7) and Human Herpesvirus 8 (HHV-8, Kaposi's Sarcoma associated Herpesvirus = KSHV). It is a characteristic of herpesviral infections that after the fulminant infection they persist in the neurons or the B- and T-cells of the body throughout life. Sometimes they reactivate. EBV, CMV and HHV-8 reproduce mainly in the B-cells of lymphotropic human herpesviruses. HHV-6 and HHV-7 reproduce in T-cells.

EBV cause most of the **mononucleosis infectiosa** diseases and can immortalize B-lymphocytes. CMV can cause **mononucleosis infectiosa** pathogenesis. A significant part of primary infections passes in an asymptomatic way. The receptor of CMV is the MHC-I class antigen, thus it can reproduce in numerous organs, which also explains the fact that it can get across the placenta, as molecules of the MHC-I supergroup are among the proteins that play an important role in the development of the placenta. Immunosuppression makes virus reproduction easier. A wide range of diseases may appear in an immunodeficient state.

HHV-6 was first described in 1986 and was isolated from patients suffering from lymphoproliferative disease. It infects CD4+ T-lymphocytes and it reproduces in them. Two variants of it HHV-6A and HHV-6B are known. Variant B is the pathogen of **exanthema subitum** (roseola infantum) and it can cause latent infection, fever, diarrhoea, nerval symptoms and hepatitis. After transplantation HHV-6 can
reactivate and it is able to replicate in liver-cells. HHV-6 can activate the replication of EBV, it reduces or increases the replication of HIV, accelerates the expression of antigens coded by HPV. HHV-7 was isolated from CD4+ cells in 1990 and it was separated from a healthy patient. It is the pathogen of pityriasis rosea, but it can also cause exanthema subitum. It can create latent infection in T-lymphocytes and productive infection in salivary gland epithelial cells. During pregnancy viremia is more frequent and may adopt urogenital, perigenital and congenital transmission. HHV-7 may play role in the cases of immunodeficient patients, in the case of immunosuppression HHV-6 and HHV-7 play the role of cofactor in states accompanied by pneumonia.

HHV-8 is the latest identified human tumorvirus, since its discovery in 1994 it has been in the limelight of research. It plays role in the AIDS associated body cavity B-cell lymphoma (BCBL). The new virus was named Kaposi's sarcoma associated herpesvirus (KSHV) by the ones who described it. Four clinical forms Kaposis sarcoma are known: 1. classical type (described by Kaposi), 2. epidemic-African type, 3. iatrogen, 4. AIDS associated type. It is possible that KSHV plays a direct or indirect stimulating role: in BCBL in elderly patients or ones suffering from AIDS, in Castleman disease, in Myeloma multiplex and in Waldenström macroglobulinaemia.

HSV-2 is the primary pathogen of primer and recurrens herpes genitalis. Asymptomatic reactivation frequently takes place due to pregnancy immunomodulation. Perinatal disease of new-born babies is rare.

**Human papillomavirus (HPV)** is one of the best-known oncogenic DNA virus. At least 95 types can be differentiated according to the sequence of E6, E7 and L1 ORF gens. HPVs are epitheliotropic viruses, they affect the epithelial cells of the skin and the mycoderm, and can cause premalign, malign metamorphosis from benign epithelial proliferation (papilla types). It is transmitted via contact, sexual and perinatal ways.

The foetus harming effect of endotoxins has been realised in the animal kingdom and in natuary. Lypid-A part in the outer membrane of Gram-negative bacteria produces the toxic effect that appear at the disgregation of the bacteria.
AIMS

1. Science has been examining the role of herpesviruses in haematologic malignant cases and in plasma-cell dyscrasia for decades now. Plasma-cell diseases (monocolanalis gammopathia) are myeloma multiplex (MM), Waldenstörm macroglobulinaemia (WM), monoclonalis gammopathy with unknown significance (MGUS). In their pathogenesis the role of HHV-8 has emerged. The increase in the number of hystocytes is characteristic of eosinophil granuloma (Langerhans cell histiocytosis, LCH). The causal role of lymphotropic herpesviruses has been suggested in the pathogenesis of LCH.

2. The aim of the work was to examine the latent presence of all four lymphotropic herpesviruses simultaneously in the patient groups listed above through serological tests. The presence of latent, reactivation and virus interactions were tested by polymerase chain reaction.

We used the blood specimen of healthy women who delivered on time without any complications as one healthy control group, in whom the reactivation of latent viruses may have been influenced by pregnancy immunomodulation. Simultaneously, samples were taken from amniotic fluids in order to be able to control viruses in the blood and in the forewaters.

The other control group consisted of people not suffering from monoclonal gammopathy, but going to the doctor with haematological disease and undergoing Non-Hodgkin lymphoma (NHL) diagnosis treatment.

3. The applied methods enable us to directly detect CMV, EBV, HHV-6 and HHV-8 DNA via simple and nested PCR. We tried to examine the blood plasma, white blood cells and the bone-marrow in order to get information about virus reactivation and the position of persistent infection. We intended to produce multiple examination on one patient in the cases of changes, mainly deterioration, which took place in the state of haematological patients or during clinical control examinations. Evidently, the examination of pregnant women was restricted to blood and amniotic fluid samples taken at the time of delivery.
MATERIALS AND METHODS

Patients and samples

Haematological patients' samples

Test samples came from of the National Center for Health (NCH), Heamatological and Stem Cell Transplantation Division. For virological examinations only the remaining samples taken for clinical tests were used, which took place only after obtaining the patients' informed consent.

Bone-marrow aspirates and blood samples taken with anticoagulant (EDTA) were processed for virological examinations and were sent by clinicians to the National Center for Epidemiology, Division of Virology.

Paraffin block samples of surgically removed granulomas were also available.

During the examination samples were taken from 69 MM-patients, 16 MGUS-patients and 10 WM-patients. The control group consisted of 44 NHL-patients. We had the opportunity for following test in the case of 38 patients. In the case of one of the 14 LCH-patients the samples of the past 17 years were available for us.

Control samples taken from healthy pregnant women during delivery

The amniotic fluids and the simultaneously taken bloods came from the NHC, Maternity and Gynaecological Department. Amniotic fluid samples were colected by disposable syringes with canueles, also used rupturing of the amniotic membrane inted of lancets. The amounts of amniotic fluid and maternal blood samples ranged betwen 4 to 10 ml. Both samples have been immediately frozen.

Method of examination

Serological examinations

EBV IgG: the detection of viral capsid antigen-specific IgG was carried out by using ELISA ("Captia™ EBV VCA (P-18) IgG" kit, Trinity, Biotech plc Bray, Ireland; or "Anti-EBV viral capsid antigen IgG EIA" kit, Diasorin s.r.l. Saluggia, Italy, catalogue number: P001606). The antigen-specific IgG detection of EBNA was carried out by using "anti-EBV Nuclear Antigen IgG EIA kit" ( Diasorin s.r.l. Saluggia, Italy , catalogue number: P001607). EBV IgM: detection of VCA-specific
IgM antibody was carried out by using "Copalis EBV-M Antibody Assay" test (Diasorin s.r.l. Saluggia, Italy, catalogue number: 315020001).

CMV serology was carried out by using the following reagents: "Enzygnost Anti-CMV/IgG" EIA kit (Dade Behring Marburg GmbH, Germany), "CMV-IgM-ELA Test PKS medac" (Medac Diagnostica Hamburg, Germany, catalogue number: 110/PKS) or "Enzygnost Anti-CMV/IgM" EIA kit (Dade Behring Marburg GmbH, Germany). The examinations were carried out strictly according to the instructions of the reagents.

Detection of HHV-6 IgG: by using "HHV-6 IgG EIA" kitel (Biotrin International Ltd. Dublin, Ireland, catalogue number: V15 HHV6).

Detection of HHV-8 antibodies was carried out as described previously (Juhász et al., 2001). HHV-8 ELISA based on a one step affinity capture of biotinylated K8.1 antigen.

**PCR (polimerase chain reaction) tests**

DNA was selected from test samples by using phenol and chloroform method. Amplification in the cases of lymphotropic herpesviruses was carried out on the basis of the publications by Mitchell et al., 1994; Aubin et al., 1993; Chang et al., 1994 és Pozo et al., 1999 with slight modifications. Products were electrophoretised in 2% agarose gel and photographed with polaroid or digital camera.

HPV specific recombinant internal control DNA was added and processed together with the sample DNA. PCR was performed with L1F and L2R primer sets amplifying HPV types. Results were considered to be unequivocal if the internal control product was present visualized by agarose gel electroforeisis. Each PCR product was hybridized with HPV group and type specific probes labelled by fluorescein.

**The sequenation of PCR products**

PCR products were cleansed/purified by using High Pure PCR Product Purification kitel (Roche Diagnostic GmbH, Mannheim, Germany) and prepared for its test on Abi Prism 300 Genetic Analyser (Applied Biosystem) by using BigDye
Terminator Cycle sequenation kit. Final DNA sequences were attached with BLAST algorhythm.

**In situ hybridization test**

In situ hybridization was used to detect HHV-6 specific DNA from the samples in 3-4µm thick paraffin-embedded sections. Samples were prepared with Paraffin Pretretment Reagent Kit for hybridization. Two HHV-6 PCR products (380 bp and 194 bp were labelled with digoxigenine-d-UTP, and applied as probe.

**The detection of bacterial endotoxin (LAL-test)**

The detection of endotoxin was carried out by using the macro method (*in vitro* semiquantitive reagent), which is a method of the LAL-test, i.e. a sensitive and specific bacterial endotoxin test.

**RESULTS AND CONCLUSIONS**

I. For the rate of occurrence of lymphotropic herpesviruses in the samples of patients suffering from monoclonalis gammopathia see Table I.

**Table I.**

<table>
<thead>
<tr>
<th></th>
<th>MM pos.</th>
<th>MGUS pos.</th>
<th>WM pos.</th>
<th>Control pos.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBV</td>
<td>69</td>
<td>36</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>CMV</td>
<td>69</td>
<td>8</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>HHV-6</td>
<td>69</td>
<td>13</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>HHV-8</td>
<td>69</td>
<td>29</td>
<td>16</td>
<td>10</td>
</tr>
</tbody>
</table>

Significant difference in the results compared to the control patient group was only realised in the occurrence of HHV-8. The difference is more noticable if the seroprevalency of the viruses is also considered. (Table II)

The seroprevalency of EBV and CMV is approximately the same in the 4 haematological patient groups. EBV was reactivated in the 60% of the patients in contrast to the much lower reactivation of CMV seropositive patients (compared to seroprevalency it is only 14).
Table II.

<table>
<thead>
<tr>
<th></th>
<th>MM</th>
<th>MGUS</th>
<th>WM</th>
<th>Control (NHL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n pos. (n)</td>
<td>pos. (%)</td>
<td>p vs control</td>
<td></td>
</tr>
<tr>
<td>EBV IgG</td>
<td>52 47 90</td>
<td>N.S.</td>
<td>12 12 100</td>
<td>27 25 93</td>
</tr>
<tr>
<td>CMV IgG</td>
<td>52 46 88</td>
<td>N.S.</td>
<td>12 11 92</td>
<td>27 18 67</td>
</tr>
<tr>
<td>HHV-6 IgG</td>
<td>52 18 35</td>
<td>N.S.</td>
<td>12 6 50</td>
<td>27 13 48</td>
</tr>
<tr>
<td>HHV-8 IgG</td>
<td>52 4 8</td>
<td>N.S.</td>
<td>12 1 8</td>
<td>26 0 0</td>
</tr>
</tbody>
</table>

1.1 Results show that KSHV acts in a different way in haematological patients and in the body of the mother due to pregnancy immunomodulation.

1.2 The rate of HHV-8 reactivation in patients suffering from monoclonal gammopathy significantly exceeds that of suffering from Non-Hodgkin lymphoma.

1.3 Virusreactivation compared to seroprevalency in the case of HHV-6 led to a result different from the case of EBV, because out of the seropositive patients the proportion of reactivation of NHL-patients and MM-patients was similar, in contrast to that of WM-patients. The appropriate proportion of pregnant is 3 out of 74 seropositive pregnant women (i.e. it was only 4%).

2. The seroprevalency of HHV-8 produced a rather surprising result. Out of 99 serological patients there were 5 seropositive, however, 30 patients produced virusreactivation or fulminant infection. This observation refers to the fact that immunosuppresion induced by HHV-8 is completely different from all other examined viruses. The phenomena is especially outstanding if the 40/95 rate found in all examined gammopathic patients is considered.

It is probable that HHV-8 serological results did not show any significant difference between the two groups because the number of seropositive patients was minimal (5 out of 99 patients).

3. Herpesvirus reactivation and infection of patients suffering from monoclonal gammopathy, the number of MM patients with concomitant herpesvirus-infections detected y PCR was the following: 15 double-, 7 triple- and 2 quadriple-positivities.
Simultaneous occurrence of acute EBV infection (IgM seropositivity) and HHV-8 PCR positivity was realised in 13 cases out of 95 gammopathiological patients.

HHV-8 reactivation may refer to immunological lesion in patients suffering from monoclonal gammopathy. During its pathogenesis, HHV-8 might play role in the formation of pathographies. EBV is proportionately present in all examined haematological pathographies. The interaction of HHV-8 and EBV might play role in the production of double infections or the pathogenesis of pathographies. The results seem to extend the results of Fleckenstein and co., who suggested the causal effect of HHV-8 in myeloma multiplex. The HHV-8 seropositivity proportion is very similar to the results of tests made among blood-donors in Hungary. The examination of healthy pregnant women shows that even pregnancy immunomodulation is not able to increase PCR-positivity above 7%. Test results of gammopathies may also be influenced by the escape of the viruses from the bone-marrow.

4. Clinicians succeeded in observing a young man (born in 1975) suffering from LCH disease for 17 years. In all his tests HHV-6 DNA was constantly present. We had the opportunity for a retrospective examination, which approved the presence of HHV-6 in the first sample when his disease was diagnosed. The clinical state of the patient improved due to antiviral therapy and the temporarily present HHV-8 and EBV disappeared from his blood. Despite HHV-6 seropositivity HHV-6 DNA could be detected all the time. The case (Figure 1) may presume the effect of persistent HHV-6 infection on other herpesviruses and on LCH pathogenesis and progression.

![Figure 1. 17-year pathography and diagnostic results of an LCH-patient](image-url)
5. Viruses and endotoxins in amniotic fluids and blood

**Endotoxins in amniotic fluids**

50 amniotic fluid samples were examined, out of which 15 amniotic fluid sample contained measurable endotoxin (0.03 EU/ml and 3.0 EU/ml). 35 samples were negative, the endotoxin value of these was below 0.03 EU/ml. The endotoxin positive samples did not contain enterovirus, which renders the pollution of samples improbable. The birth weight of the foutsuses was not lower than the average, which does not imply fulminal metabolism impairment. There should be an opportunity to follow the development of new-born babies in order to be able to detect further lesions (kidneys).

**Lymphotropic herpesviruses in amniotic fluids**

106 amniotic fluid samples were examined. Herpesvirus DNA was detected in 27 samples. CMV DNA was detected in 9 samples, HHV-7 DNA in 8, HHV-8 DNA in 5 and EBV DNA in 4 samples. HHV-6 DNA was only found in one sample.

**Papillomaviruses in amniotic fluids**

Papillomaviruses do not spread via blood, so its examination only had to be carried out in amniotic fluid samples. Out of 96 7 samples were positive. 18, 39, 45, 58, 62 HPV genotypes and one non-typable, that is not anogenital type was detected. The possibility of contamination at sample-taking was excluded by the negative result of the HHV-2 PCR test. HPV type 39 has not been detected in Hungarian patients by other authors so far. Our data reinforce our earlier observations that HPV can spread not only in a perinatal way but also vertically.

**Influence of endotoxins and viruses and mean weight of new-born babies**

The average weight of new-born babies was defined in both the virus positive and endotoxin positive and the virus negative and endotoxin negative groups. The average weight of male and female new-born babies served as control. There was no difference between endotoxin positive and virus positive new-born babies compared to the average. The six twins were exceptions whose weight was not included in the averages.

**Lymphotropic herpesviruses in blood**

Lymphotropic herpesviruses present in both the white corpuscles and in the plasma were detected in 106 maternal hemolytic blood samples. There were 7 HHV-8
positive, 3 HHV-6 positive, 1 EBV positive, 1 CMV positive and 1 HHV-7 positive samples. The number of positive results simultaneously present in the amniotic fluid and the blood was only 2 out of 106-106 samples. The rate of occurrence of CMV, HHV-7 and EBV was 2-4 times higher in the amniotic fluid than in the blood. Further tests are necessary to be able to see whether immunotolerance can be induced by the transmission of the virus through the placenta in foetuses. The adverse behaviour of the HHV-8 serological results and the PCR results might refer to the development of immunotolerance.

**Anti-HHV-6 IgG antibodies in the maternal blood**

HHV-6 antibodies were detected in the 70 % of the blood of mothers (74 samples). There were two samples in which there was no antibody, however, HHV-6 DNA was detected in the blood of one of them and in the amniotic fluid of the other

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**LIST OF PUBLICATIONS**

**Publications served as basis for the thesis**

**Articles:**


Ádám Fazakas, Márta Csire, György Berencsi, András Matolcsy, Lajos Jakab, István Karádi, Judit Várkonyi 2006: „Human Herpes Virus-6 induced coexistent POEMS and Castleman Disease” Pathology Oncology Research, Manuscript ID:655/POR (under revision)


Other publications

Book chapters:


Articles:


**Popular publications**


Berencsi György, Csire Márta, Younes Saleh Ali 2005. „Szexuális úton terjedő vírusfertőzések II.” Paramedica, IV. évf., 4. szám Hungarian

Berencsi György, Csire Márta, Younes Saleh Ali 2005. „Szexuális úton terjedő vírusfertőzések és következményeik. III. Paramedica Egészségmagazin IV. évf. 5 szám. Hungarian

**Oral presentations, posters and their abstract that appeared in scientific journals (Abst):**


Istvan Valyi-Nagy, Marta Csire, Gabor Mikala, Attila Juhasz, Monika Peto, Judit Janosi, Janos Jako, Gyorgy Berencsi 2003: „Detectability of HHV-8 Infection in Multiple Myeloma Patients in Hungary – Effect of Disease stage and Treatment
History” The American Society of Hematology 45th Annual Meeting and Exposition December 5-9, 2003 San Diego, California, USA
Abst.: # 5183 appears in Blood, Volume 102, issue 11, November 16, 2003
Abst.: # 4907 appears in Blood, Volume 102, issue 11, November 16, 2003
Abst.: Magyar Belorvosi Archivum LV.évfolyam 3/2002 75.oldal, Hungarian
Abst.: Magyar Belorvosi Archivum LV.évfolyam 3/2002 96.oldal, Hungarian
Abst.: Magyar Onkológia 46.évfolyam 1.szám 2002. 90.oldal. Hungarian
Abst.: Magyar Onkológia 46.évfolyam 1.szám 2002. 98.oldal.


