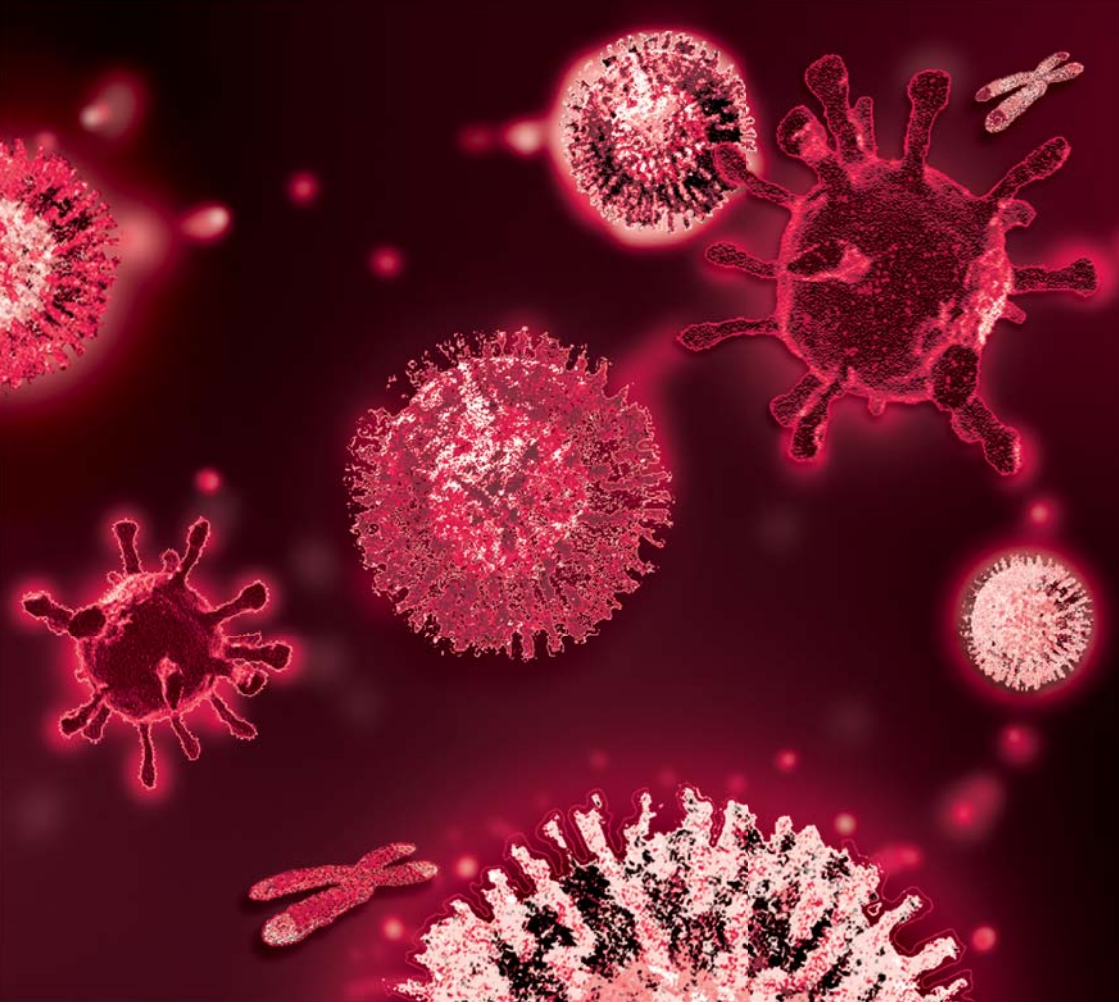


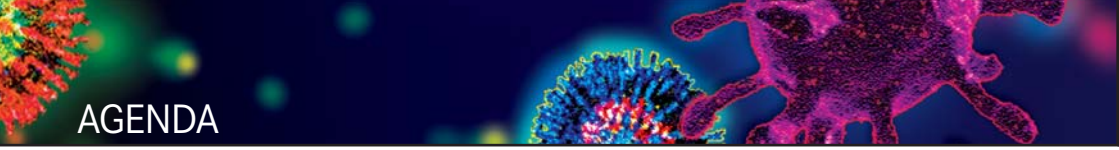
RĪGA STRADIŅŠ UNIVERSITY

AUGUST KIRCHENSTEIN INSTITUTE OF MICROBIOLOGY AND VIROLOGY

Workshop

IMMUNOMODULATING HUMAN HERPESVIRUSES AND THEIR ROLE IN HUMAN PATHOLOGIES

AGENDA | PRESENTATIONS SUMMARIES | OCTOBER 13–14, 2011



AGENDA

Workshop **IMMUNOMODULATING HUMAN HERPESVIRUSES AND THEIR ROLE IN HUMAN PATHOLOGIES**

October 13, 2011

10:00–10:10 **Welcome**
(Uldis Berkis, Head of the Research Department)

Session Chairs: Dr.med. Sandra Lejniece
Dr.med. Modra Murovska

10:10–10:50 **Immunomodulating properties of HHV-6 and HHV-7:**
- **Immunosupressive effects of HHV-6 and HHV-7: clinical importance**
(Dr.med. Simona Donina)
- **HHV-6 and HHV7 similarities and differencies**
(Dr. Inta Jaunalksne)

10:55–11:15 **Herpesviruses Infection and Diseases of Nervous System**
(Dr.med. Inara Logina)

11:20–11:40 **Role of beta-herpesviruses infection in the development of Chronic Fatigue Syndrome/Myalgic Encephalomyelitis**
(BSc Santa Rasa)

11:45–12:05 **Coffee Brake**

12:05–12:25 **Assessment of HHV-6 and HHV-7 in Patients after Kidney Transplantation: Impact on Clinical and Immune Parameters**
(Dr.med. Inese Folkmane)

12:30–12:50 **Relationship between beta-herpesviruses reactivation and complications development after peripheral blood stem cell transplantation**
(Dr. Ilze Trociukas)

12:55–13:15 **Presence of HHV6A, HHV6B and EBV DNA in peripheral blood of primary chronic lymphocytic leukemia patients and its influence on B-cell ubpopulation profile**
(Dr. Artjoms Spaks)

13:20–14:20 **Lunch Brake**

Session Chairs: Dr.med. Inara Logina
Dr.biol.sci. Mykolas Mauricas

14:20–14:40 **Cellular Immune Respons in HHV-6/-7 Infected Colorectal Cancer Patients**
(Dr.med. Simona Donina)

14:45–15:05 **Effect of General and Regional Anaesthesia on Activation of Beta-Herpesviruses, Immune Response and Postoperative Period in Prolonged Reconstructive Surgeries**
(Dr. Arnis Vilks)

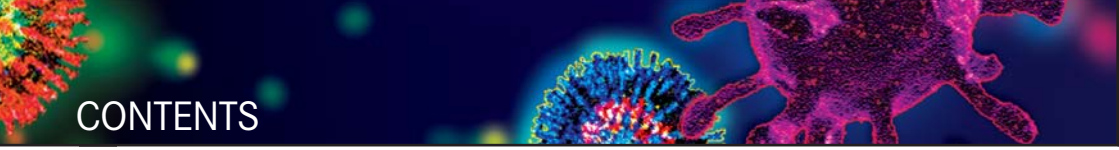
15:10–15:30 **Frequency of HHV-6 and HHV-7 Infection Markers in Rheumatoid Arthritis and Osteoarthritis Patients and Changes in Cytokine Expression Levels**
(Dr. Anda Kadisa)

15:30–15:45 **Discussion**

October 14, 2011

Session Chairs: Dr.med. Simona Donina
Dr.med. Vaira-Irisa Kalnina

10:00–10:40	**Role of T-cell subsets in regulation of immune response** (Dr.med. Dainius Characiejus)
10:45–11:05	**Occurrence of human herpesviruses 6 and 7 infection in Belarus** (Cand.biol.sci. Svetlana Orlova)
11:10–11:30	**Association of HHV-6 and HHV-7 with Diseases of Thyroid Gland** (Dr.med. Zaiga Nora-Krukle)
11:35–11:55	**Coffee Brake**
11:55–12:15	**Usage of Natural Glycopeptide for Treatment of Herpes Viruses Infection** (MSc Guntis Vitols)
12:20–12:35	**Antiviral agents active against HHV-6 and HHV-7** Dr.med. Vaira-Irisa Kalnina
12:35–12:55	**Discussion and Closing**



CONTENTS

IMMUNOSUPRESSIVE EFFECTS OF HHV-6 AND HHV-7: CLINICAL IMPORTANCE

Simona Donina
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REVIEW OF LITERATURE

Both the A and B variants of HHV-6 enter the cell through interaction with CD46, which is a ubiquitous type glycoprotein expressed on the surface of all nuclear human cells [1]. CD46 is a member of a family of glycoproteins acting as regulators of complement activation. HHV-6 is a lymphotropic virus, but as it uses CD46 as a cellular receptor, it may also infect other cells, such as monocytes, glial cells, endothelial and epithelial cells [2]. HHV-6 is capable of persistence in the host after primary infection. Candidate sites for latency are monocytes [3] and early bone marrow progenitor cells [4], whereas salivary glands and brain tissue are suspected for harbouring persistent HHV-6 infection [5]. HHV-6 can reactivate by superinfection with HHV-7 [6].

HHV-6 has potential immunomodulating properties as the virus can induce proinflamatory cytokines such as TNF-alfa, IL-1 beta, IL-10, IL-12 in monocytes/macrophages and downregulate CD3 in infected T cells [7]. In addition, infection of PBMC has been reported to suppress T cell functions, including IL-2 synthesis reduction and cell proliferation [8]. The ability of HHV-6 to alter the expression of these key immune activation molecules lead to a changed pathogenesis associated with infections. Cell mediated immunity against HHV-6 is a critical element of the host defence. Individuals with defects in NK cell function are suspectable to herpesvirus infections. HHV-6 infection of NK cells results in increased production of IL-15 that both increases NK-mediated killing of HHV-6 and stimulates IFN-gamma production from CD4 T lymphocytes and NK cells [9, 10]. HHV-6 could induce CD4 T lymphocyte depletion. It was clearly demonstrated in mice models; thymocyte depletion was induced in directly infected cells in HHV-6A or HHV-6B infected mice [11]. Progressive destruction of the thymus could induce T lymphocyte depletion by affecting T lymphocyte development. HHV-6 induces apoptosis in the CD4+ T cell line *in vitro*. The majority of apoptotic cells are uninfected cells and apoptosis induction is independent of viral replication; TNF-alpha and Fas cross-linking enhance the observed apoptosis [12]. HHV-6A can also deplete CD4 T lymphocyte number by inducing CD46-mediated cell fusion [13]. HHV-6 also decreases PBL proliferation via transcriptional down-regulation of Il-2 in mitogen-stimulated purified T lymphocyte populations *in vitro* [14]. In addition, HHV-6 down-regulates CD3 transcription [15].

It has been found that HHV-6 induces type I response in T lymphocytes [16]. Among the genes up-regulated were IL-18, the IL-2 receptor and members of the TNF receptor superfamily. HHV-6 down-regulates the chemokine receptor CXCR4 in directly infected CD4+ cells [17].

A clinical correlation between immune response affecting effects is found in evidence that HHV-6 reactivation may delay platelet engraftment and cause neutropenia in stem cell transplant recipients [18]. These effects may contribute to organ-specific inflammation during HHV-6 infection as well. HHV-6 causes lymphocyte infiltration and increases the expression of adhesion molecules ICAM-1 and VCAM-1 in the solid organ transplant, which may lead to the local inflammation and graft damage and may even trigger allograft rejection [19]. HHV-6 infection after transplantation has been associated with other opportunistic infections, particularly of fungal origin, but also viral infections may occur [20, 21]. HHV-6 has been postulated as a modifier of CMV replication, given evidence that HHV-6 stimulates secretion of TNF-alpha [22]. Clinical data on the interaction between HHV-6, CMV and HCV are mainly from transplant population. Studies of renal and liver transplant patients showed an association between seroconversion, detection of HHV-6 DNA in PBMC or detection of HHV-6 and CMV DNA in either urine or serum, and CMV infection, reactivation, viral load, disease and disease severity [23, 24].

HHV-7 was first isolated from CD4+ T cells obtained from a healthy adult in 1990 [25]. The virus primarily infects T cells and it uses CD4 as a cellular receptor [26]. After primary infection HHV-7 establishes latency in the host and may reactivate under immunosuppression. Little is known about the preferred sites for HHV-7 replication, but causes of encephalitis and hepatitis have been reported. Despite extensive clinical evaluation, little evidence that the virus causes lytic infection exists. Interaction of HHV-6 and/or HHV-7 with CMV and the immune modulating properties are the most important factors in the illness-causing potential [27, 28, 29].

HHV-6 infections in immunocompetent individuals are self-limiting, whereas in immunocompromised patients reactivation of latent virus may cause serious complications. The degree of immunosuppression and reactivation of beta-herpesviruses are often tightly linked, so unassailable proof of causation is elusive. Conclusive proof requires selective suppression of the virus in a randomized trial. Such studies have been performed on solid organ transplant and stem cells transplant patients, and showed a decreased incidence of bacterial and fungal infection in the group receiving CMV prophylaxis, providing evidence for a CMV-induced immunosuppressive effect. No selective suppression trials have been performed for HHV-6 or HHV-7 [29].

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HHV-6/HHV-7 SIMILARITIES AND DIFFERENCES

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HHV-6 is a member of the Betaherpesvirinae (subfamily of the Herpesviridae) which also includes HHV-7 and cytomegalovirus (HHV-5 or HCMC). HHV-6 has two subtypes – HHV-6A and HHV-6B. These two variants are highly distinctive, diagnostic tests are usually unable to distinguish these infections. Cells infected with HHV-6B lose possibilities of DNA synthesis within 65 hours of infection. HHV-7 is recently described as a T lymph tropic herpesvirus. HHV-7 is similar to human HHV-6 in its genetic content and in many of its biological properties, but there are differences between HHV-7 and HHV-6. HHV-7 binds to cellular CD4 molecule and uses this protein as a necessary component of its receptor, but HHV-6 binds to a different receptor – CD46. HHV infects lymphocytes, monocytes, epithelial, endothelial cells, fibroblasts, glioblastoma, fetal astrocytes, oligodendrocytes. CD8+, gdT cells and NK cells from lymphocytes can be infected. HHV could persist at low levels in cells and tissues (1,4,5,6).

Both HHV-6 and HHV-7 are found in human saliva. The transmission of the virus is not well known, but the most common way is through saliva. There is a point that salivary glands act as a container for persistent or latent viral infections, therefore virus is replicated in salivary glands and secreted in saliva, which works as a transmission way. There

have been suggested other routs of transmission. Transmission trough breast feeding is also doubtful since HHV-6 DNA and HHV-7 was not found in breast milk (5).

Integration of HHV-6 genome in lymphoblasts from leukemic patients and his offspring raised the possibility of genetic transmission. A vertical transmission was not observed in other cases of genome, the presence of HHV-6 DNA offspring was interpreted as a tendency of HHV-6 to integrate at specific chromosomal loci (5).

Replication in salivary glands is observed for HHV-6B, but not for HHV-6A. Lymphocytes and monocytes represent two known sites of latent infection reservoir.

HHV-6, HHV-7 can cause mild to fatal illnesses and can live commensally on its host. HHV-6 primary infections account for up to 20% of infant emergency visits for fever and are associated with several more severe complications, such as encephalitis, lymphodenopathy, myocarditis, myelosupression, pneumonitis. The prevalence of the virus in the body increases with age. Infection rates are highest among infants between 6–12 month old and it's hypothesized that this is due to loss of maternal antibodies in a child that protects him from infection. After primary infection latency is established in myeloid and bone marrow progenitors. It exists for the lifetime of the host. HHV-6B is responsible for 93% of primary infections, such infections usually cause fever with exanthema subitum (roseola infantum), also known as roseola infantum or sixth disease, only being observed in 10% of cases. HHV-7 may also cause fever with or without rash. HHV-7 is acquired later in life than HHV-6 (after the age of 3). There is no apparent disease in late childhood that could be associated with an acute HHV-7 infection. Acute HHV-7 infection has been associated with hepatitis. The most likely conclusion is that primary HHV-7 infection is not associated with a definable syndrome that acquires HHV-7 infection after 2 years of age. HHV-7 infection remains poorly understood (8.9).

Primary infections in adults are rare since most occurrences are in children. When infection occurs for the first time in an adult, the symptoms can be severe. The virus periodically re-activates from its latent stage. Reactivation is often asymptomatic, but in immunocompromised individuals there can be serious complications – for example, in transplant recipients it can lead to graft rejection, in HIV/AIDS – HHV-6 reactivation causes disseminated infections leading to end organ disease and death (3).

Although up to 100% of the population are exposed (seropositive) to HHV-6, most before reaching 3 years of age, there are rare cases of primary infections in adults. But if they occur – these have been linked more with HHV-6A, which is thought to be more pathogenetic and more neurotropic and has been linked to severe central nervous system related disorders.

HHV-6 has been reported in MS patients and has been implicated as a cofactor in chronic fatigue syndrom, fibromialgia, AIDS and temporal epilepsy (2, 6.) There is information that HHV-6 and HHV- 7 infection may increase the Graves disease in individuals with inherited diminished TP 53 apoptotic function (7). HHV-6/HHV-7 is also associated with Kaposi sarcoma. HHV-7 tropism is associated with T lymphocytes, CD68+ cells. U 83 encoded by HHV-6 may contribute to disregulation of cellular expression of signalling and it may play a role in tumour progression.

HHV-6 and HHV-7 similarities:

- both viruses are related to the subfamily of Herpesviridae
- genetic content and biological properties are similar
- both live primary in humans, infect lymphocytes, neuronal cells
- the main transmission way is through saliva
- both cause infection in early childhood
- after primary infection latency is established
- reactivation is caused by immunosuppressant
- reactivation result depends on immunodeficiency degree.

HHV-6 differences:

- there are 2 subtypes: HHV-6A and HHV-6B
- HHV-6 binds to CD46
- replication in salivary glands is observed for HHV-6B, but not for HHV-6A; it is more neurotropic

- in early childhood infections are caused by HHV-6B
- HHV-6A is related to adults primary infection and is more neurotropic related to central nervous system disorders
- HHV-6A is detected in monocytes and lung tissue.

HHV-7 differences:

- HHV-7 binds to CD4
- causes infection in an early childhood, but occurs later than HHV-6B infection
- clinically mimics HHV-6B infection, it is not so well characterized
- HHV-6 or HHV-7 subtypes are associated with different disease pathogenesis.

Better recognition and detection in clinical setting of HHV-6 and HHV- 7 could lead to different pathological processes.

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HERPESVIRUSES INFECTION AND DISEASES OF NERVOUS SYSTEM

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At least eight virus types of the heterogeneous family of *Herpesviridae* are known to infect men frequently. The first 3 types or *Alphaherpesvirinae* subfamily – *Herpes simplex*-1 and -2 (HSV-1, HSV-2) and *Varicella Zoster* viruses (VZV) are acknowledged as typical neurotropic and neuroinvasive viruses. They persist in the body by becoming latent

and hiding from the immune system in the cell bodies of nerves, notably in neural ganglia (30). HSV-1 tends to reside in the trigeminal ganglia, HSV-2 – in the sacral ganglia, while VZV – in the trigeminal ganglia and higher spinal region (cervical, thoracic), however, these are only tendencies and not the absolute rule. In an outbreak, the virus in a nerve cell becomes active and is transported via the nerve' axon to the skin, where the virus replication occurs, causing new sores or shingles, however, it is possible that the dissemination of the virus occurs in another direction, e. g., the central nervous system – to brain or spinal cord. The cell tropism of *Betaherpesvirinae* subfamily – Cytomegalovirus (CMV), human herpesviruses 6 and 7 (HHV-6, HHV-7) are linked and traced in the peripheral mononuclear blood cells (PMBC), but there are studies detecting these viruses in different other tissues, like cerebrospinal fluid, normal and encephalitic brain from biopsies or post-mortem studies (10; 11; 12; 21); likewise, there is research on different neurotropism of HHV-6A, HHV-6B (18), however, HHV-7 can be found in brain tissue at a lower frequency than HHV-6 (11). Cell tropism of Epstein-Barr virus (EBV) from *Gammaherpesvirinae* subfamily is related to B lymphocytes.

After initial or primary infection with human herpesviruses in one's childhood and its lifelong latency some infected people experience sporadic episodes of viral reactivation or outbreaks, in many cases manifesting as mild skin reactions that are resolved spontaneously. However, they have been associated with different neurological manifestations in infants and adults, particularly immunocompromised. There are known several pathological conditions in the development of nervous system which have been initiated by the influence of human herpesviruses: shingles, postherpetic neuralgia and peripheral nerve damage, neuroinfections, immune-mediated demyelinating inflammatory diseases of the central and peripheral nervous system (CNS and PNS), as well as nowadays there are reports linking viruses with some neurodegenerative and paroxysmal states.

Skin lesions caused by herpesviruses are very typical manifestations of initial infection or latent viral reactivation – cold sores and other herpetic vesicles by HSV-1 and HSV-2, chicken pox and herpes zoster by VZV, *exanthema subitum* by HHV-6 and HHV-7. Viruses can reactivate under stress or immune suppression. The recurrence of VZV replication especially and sometimes HSV-1, -2, which persist lifelong in neural ganglia is accompanied by severe radicular pain in discrete areas, those innervated by the corresponding nerve in which the latent infection has occurred. A few days later herpes zoster lesions occur in restricted areas (dermatome) that are innervated by this single ganglion. Reactivation can affect the eye via the trigeminal nerve (uveitis, keratitis, conjunctivitis, ophthalmoplegia, iritis) and the brain via the cranial nerve VII and VIII (Bell's Palsy and Ramsay-Hunt syndrome). Reactivation can lead to postherpetic neuralgia – chronic burning or itching pain, associated with increased sensitivity to touch (allodynia, hyperesthesia). The pain may last well after the rash has healed (even months or years).

Herpesviruses as infectious agents play a role as an etiological factor in neuroinfections – a group of the devastating infectious diseases affecting the nervous system in children and adults worldwide (32). The burden of disease is high as survivors may have neurological *sequelae* affecting motility, sensory organs and mental functions, or develop epilepsy, thus ending in precarious states for the rest of their lives. Many aspects of management of neuroinfections depend on its categorization according to the causative infectious agent (viral, bacterial, parasites), the main sites of inflammation (meningitis, encephalitis, myelitis, neuritis, etc.), the mode of pathogenesis (primary or secondary) as well on geographical distribution of infectious vectors. Encephalitis is defined by the presence of an inflammatory process of the brain parenchyma in association with clinical evidence of brain pathology – cognitive dysfunction, behavioural changes, focal neurological abnormalities and seizures accompanied by a febrile disease, headache and altered level of consciousness. HHV-1 (rarer HHV-2), VZV, EBV, CMV are commonly approved causes of infectious viral encephalitis, also HHV-6 can be responsible (16; 32), including febrile convulsions in complicated *exantema subitum* and encepahalitis in infants and immunocompromised (4; 13; 19; 25). HHV-7 has not been traced to cause a specific disease, but is associated with febrile convulsions and encephalitis (9). HSV encephalitis and CMV periventriculitis and myeloradiculitis include a prominent necrotizing component, mainly seen in HIV patients. CNS complications of VZV, CMV and HSV include other localizations as well: myelitis, arteriitis, ventriculitis, meningitis (16; 32). Primary encephalitic processes may secondarily involve the meninges with inflammatory infiltration resulting in symptoms of meningeal irritation

(headache, photophobia, nucheal rigidity, nausea) and usually mild CSF pleocytosis. Viral meningoencephalitis implies a viral infection process of both the brain/spinal cord and the meninges. Isolated aseptic meningitis is an uncommon manifestation of human herpes viral infection, however, most commonly because of HSV-2.

Nowadays a lot of data have been accumulated on the immunomodulating properties of herpesviruses, the regulating influence on the host cell genome and interference with natural cell death mechanisms and other factors. The most studied immune-mediated diseases of the nervous system are multiple sclerosis, acute disseminated encephalomyelitis and postinfectious polyradiculoneuritis or Guillain-Barre syndrome (GBS). Acute disseminated encephalomyelitis (ADEM) is the immune-mediated condition with multiple small demyelinated foci around small veins of the white matter, featuring cellular infiltration composed by lymphocytes, macrophages and microglia (5). ADEM is an autoimmune disease with an evidence of cell-mediated immunity to the myelin basic protein. Unlike multiple sclerosis ADEM is monophasic disorder with rapidly progressive course, on the other hand sometimes it is hard to distinguish it from acute viral encephalitis.

Multiple sclerosis (MS) is a central nervous system disorder marked by decreased nerve function with initial inflammation of the protective myelin nerve covering and eventual scarring. MS symptoms and their severity varies a lot (motor weakness, vertigo and disbalance, optic and oculomotor symptoms, sensory disturbances, behavioural and cognitive signs, etc.) and they may progress into episodes of crisis alternating with episodes of remission. It is generally accepted that MS is a systemic T-cell-mediated autoimmune disease (22) with a T-helper type 1 (Th1) profile of cytokine production (6); however, the precise etiology of MS remains unknown. Viruses have long been proposed to be either initiating factors for MS or directly pathogenic in the development of MS (7). Recently much attention has been paid to the relationship between HHV-6 and MS. HHV-6 antigens are expressed in the nuclei of oligodendrocytes in inflammatory lesions of brain tissue (8). IgM and increased IgG specific anti-HHV-6 antibody titres are detected in plasma/serum (1; 2; 31) and cerebrosipnal fluid (2). HHV-7 is similar to HHV-6 in its genetic content (26), which includes the possible association with MS.

We studied 67 (48 females and 19 males) patients with MS diagnosis confirmed clinically by MRI and laboratory tests – 36 had remitting/relapsing (RR) MS type, 26 – secondary progressive (SP) MS type and 5 – primary progressive (PP) MS type (28). Activation of HHV-6 and HHV-7 infection was detected in correlation with exacerbation of MS that was approved by acute lesions detected in the patients with MS by MRI. HHV-6 DNA in blood plasma (active infection) was detected in 12 of 45 (26.7%) patients with MS by PCR, but not in any of the plasma samples from the control group patients with other non-demyelinating neurological diseases (OND) or the blood donors. HHV-7 genomic sequence was found in 51/68 (75.0%) peripheral blood leucocytes (PBL) DNA samples and in 15 (29.4%) plasma samples from the patients with MS with statistically significant difference between the patients with OND ($p=0.00036$) and the blood donors ($p=0.00049$). In order to confirm PCR results, the patients' blood and plasma was checked for HHV-6 and HHV-7 specific IgG and IgM antibody presence. The patients with SP/MS and PP/MS had similar results. Totally in 26.7% MS patients HHV-6 specific and in 29.4% – HHV-7 specific IgM antibodies were present. Part of MS patients were examined during periods of exacerbation and remission. The viremia and virus specific IgM antibodies were found only during disease exacerbation, including the presence of Gd-enhancing lesions on MRI, and not during remission or relative remission with an absence of Gd-enhancing lesions on MRI. Overall we found active HHV-6 infection during exacerbation of RR/MS in 23.1% of all cases, during exacerbation of SP/MS – in 31.3% of the patients; active HHV-7 infection during exacerbation of RR/MS – in 28.0%, during exacerbation of SP/MS – in 28.6%. Thus, we conclude that HHV-6 and HHV-7 activation does not depend on MS course type. It revealed that proinflammatory cytokines IL-12 and TNF-α are involved in the pathogenesis of MS. We found increased IL-12 and TNF-α production in MS patients, but not in the OND control group patients. A correlation between the concentration of IL-12 and TNF-α in the plasma and RR/MS flare-ups was discovered, which is proven by MRI-diagnosed active inflammatory damage. A higher IL-12 level in RR/MS patients is found only in disease exacerbation periods; also, the TNF-α concentration in the patients' plasma is considerably higher during flare-ups than during periods of remission ($p=0.001$). Patients with SP/MS show a higher

plasma concentration of IL-12 and TNF-α in both relative remission periods and during flare-ups, however, during flare-ups the IL-6 concentration is 1.8 times and the TNF-α concentration is 3 times greater (p=0.001). During MS clinical flare-ups there is an increased IL-12 and TNF-α plasma concentration in those patients who have an active HHV-6 and/or HHV-7 infection; this leads to suggest that virus reactivation has a cytokine synthesis-stimulating effect *in vivo*.

HHV-6 and HHV-7 infect immune system cells and can change the functioning of these cells. The correlation between HHV-6 and HHV-7 reactivation, elevated concentrations of IL-12 and TNF-α in plasma and RR/SM and SP/MS exacerbation indicates to the viruses involvement in disease pathogenesis.

Autoimmune inflammatory demyelinating neuropathies are a group of disorders in which the body's immune system attacks a part of the peripheral nervous system. Guillain-Barre syndrome (GBS) is one of the best known and described form of these diseases. The first symptoms of this disorder include varying degrees of weakness or tingling sensations in legs, in many cases spreading to arms and upper body. In severe cases the patient is almost totally paralyzed and the disorder is life threatening – potentially interfering with breathing and autonomic regulation. Pathology in GBS is attributed to sensitized T cells mediated autoimmune response directed to peripheral nerve sheaths and myelin involving macrophages, cytokines and autoantibodies produced by B lymphocytes. GBS is often preceded by a bacterial or viral infection – *Campylobacter jejuni, Mycoplasma pneumonia* or CMV, EBV. GBS association with HHV-6 has been mentioned, too (23), but HHV-7 association with diseases is not reported widely. The major part of information is case reports. Similar etiopathogenesis, which is tied with autoimmune mechanisms, is supposed to be chronic inflammatory polyneuropathies (17). Cellular and humoral mechanisms are involved in the development of autoimmune inflammatory demyelinating peripheral neuropathies.

Investigations carried out in our laboratory to check HHV-6 and HHV-7 association with the nervous system demyelinating diseases and the data obtained about association of these diseases with HHV-7 reactivation allowed suggesting that HHV-7 can be associated with demyelinatig processes (33). In the latest study (28) of 45 GBS patients we found that HHV-6 and HHV-7 infections were associated with acute demyelinating polyneuropathies – HHV-6 active infection was detected in 25.0% of the patients with GBS, active HHV-7 infection in 41.0%, making statistically a significant difference between patients and blood donors; p=0.000077.

There are no published data on relationships between HHV-6 and HHV-7 and a mechanical damage of the nervous system – vertebrogen radiculopathies (VR); the latter is of great clinical interest due to the significantly large proportion of patients with back pains in general practice. Frequent is also failed back surgery syndrome, i. e., recurrent pain after invasive treatment of pain. This is the reason for the increasingly popular opinion that factors which cause infectious processes are also significant in radicular pain syndrome, neurotropic viruses being among them. Latent/persistent HHV-6 infection with no statistically significant difference was observed in our study in both non-operated patients (n=16) with VR (50.0%), operated patients (n=24) with VR (45.8%), and practically healthy blood donors (28.7%) in the control group. Latent/persistent HHV-7 infection was also found in 81.25% of the non-operated patients with VR, in 79.2% of the operated patients with VR, and 75.3% of the practically healthy blood donors. Active HHV-6 infection was found in 25.0% of the non-operated patients with VR, but was not found in the practically healthy blood donors under observation (20). HHV-7 viremia was significantly more frequent in the non-operated patients with VR (38.5%; p=0.0161) and the operated patients with VR (52.6%; p=0.00008) than in the practically healthy blood donors (10.6%). In the cases of virus genomic sequence detected in DNA of peripheral blood cells, the presence of IgG and IgM specific antibodies were not found. All samples with an active viral infection approved by nPCR had both IgM specific viral antibodies and elevated IgG titer. Clinical and radiological examination of the non-operated patients with VR during exacerbation of pain revealed that both patients with active HHV-6 infection and 5 patients with HHV-7 infection had Gadolinium-enhancement on MRI, implying active local inflammation.

Our study confirmed the possible relationship of HHV-6 and HHV-7 to autoimmune inflammatory demyelinating and non-demyelinating CNS and PSN diseases, as well as the relationship between the virus activation and the clinical activity of the diseases. This is especially important because there has been no strong evidence on the role of HHV-7 in

infectious demyelinating polyneuropathies and vertebrogen radiculopathies. Active HHV-7 infection is significantly more frequent in both mentioned groups of patients with PNS damage than in blood donors (p=0.000031 and p=0.00002 respectively) and in MS patients (29.4%; in GBS patients – in 41.0%).

Testing for the HHV-6 variants was performed on all patient groups studied. Patients with MS and patients with PNS diseases had HHV-6B variant in PBL. The data in literature reveal that the HHV-6B variant is more frequently found in the PBMC cells of MS patients (1), but that in cells of free serum the HHV-6A variant is present more often (3).

More and more reports have been published not only about the infectious and immunomodulating properties of herpesviruses, but also about the regulating influence on the host cell genome. HSVs may persist in a quiescent but persistent form, known as latent infection, notably in neural cells (30). During such latent infection of a cell, HSVs express Latency Associated Transcript (LAT) RNA. LAT is known to regulate the host cell genome and interfere with natural cell death mechanisms. In the presence of a certain gene variation (APOE-epsilon4 allele carriers), a possible link between HSV-1 and Alzheimer's disease was reported as early as in 1979 (24). HSV-1 appears both to be particularly damaging to the nervous system and increases one's risk of developing Alzheimer's disease. The virus interacts with the components and receptors of lipoproteins, which may lead to the development of Alzheimer's disease (14; 29). Without the presence of the gene allele, HSV-1 does not appear to cause any neurological damage or increase the risk of Alzheimer's. There circulate ideas that HHV-7 also might be linked to the development of neurodegenerative diseases. Several studies stated the association with HHV-6 and potential pathogenetic mechanisms of mesial temporal lobe epilepsy (15; 27).

Human herpesviruses are the hidden neighbours for majority of people lifelong and worldwide, however, for some of them they might turn into dangerous tools and can cause serious diseases of the nervous system.

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ROLE OF BETA-HERPESVIRUSES INFECTION IN THE DEVELOPMENT OF CHRONIC FATIGUE SYNDROME/ MYALGIC ENCEPHALOMYELITIS

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INTRODUCTION

HHV-6 and HHV-7 are ubiquitous and immunomodulating viruses that replicate mainly in CD4+ T-lymphocytes, but they infect a variety of human cells, such as lymphocytes, monocytes/macrophages, natural killer cells, dendritic cells (Krueger & Ablashi, 2003; Otani & Okuno, 2007). After primary infection, both viruses establish latency. They can be reactivated in some conditions, such as an immunosuppressed state and are relevant pathogens in immunocompromised hosts (Richman, et al., 2002).

Chronic fatigue syndrome/myalgic encephalomyelitis (CFS/ME) is defined as severe, unexplained, chronic fatigue lasting at least six months, accompanied by four of eight symptom criteria: sore throat, tender cervical or axillary lymph nodes, muscle pain, impaired memory or concentration, un-refreshing sleep, post-exertional malaise, headaches (Fukuda, et al., 1994). The illness starts suddenly with an infectious-like symptom. Various viral and microbial infections have been implicated as possible triggers of CFS/ME, including human herpesvirus-6, Epstein-Barr virus, enteroviruses, parvovirus B19, and Lyme disease and Q fever causing bacteria (Komaroff, 2006). Recently there has been reported finding of xenotropic murine leukemia virus related virus (XMRV) in 67% of persons with CFS/ME and 3.7% healthy controls (Lombardi, et al., 2009). In the meantime, other studies failed to detect XMRV in patients with CFS/ME and controls (Shin, et al., 2011). CFS/ME, which came to attention in 1988, is a complex and incompletely understood illness. International Consensus Panel developed criteria and suggest to use the term "myalgic encephalomyelitis" (ME), due to its widespread inflammation and multisystemic neuropathology (Carruthers, et al., 2011). Data have been published supporting a heritable predisposition to CFS/ME with a significantly elevated risk among the first, the second and the third degree relatives of CFS/ME cases. Predisposition could have a genetic basis, however, environmental factors, viral illnesses, stressful life and traumas could also be involved (Jason, et al., 2000, Jason, et al., 2005-2006). According to the population based studies, the estimated worldwide prevalence of CFS/ME is 0.4 – 1%. CFS affects approximately 17 million people, including over 800 000 people in the US and about 240 000 – in the United Kingdom (Lorusso, et al., 2009).

Reactivation of HHV-6 and HHV-7 had been detected in patients with CFS/ME and it could be a result of latent herpesvirus infection reactivation (Chapenko, et al., 2006; Krueger & Ablashi, 2006). Other studies suggested that the frequency of HHV-6 and HHV-7 reactivation can be an objective biomarker for fatigue (Kondo, 2007). Although the Center for Disease Control and Prevention (CDC) study found no association between CFS/ME and infection by such human pathogens as human herpesvirus-6, Epstein-Barr virus, enteroviruses, human retroviruses, rubella virus, *Candida albicans*, bornaviruses and mycoplasma. CFS/ME could be supposed to have multiple causes and some viruses or infectious agents might contribute a subset of CFS/ME (CDC, 2010). Still there are no effective standardized and

reproducible diagnostic tests, medical treatment and prevention strategies of CFS/ME; hence the etiology, risk factors, and pathophysiology of CFS/ME remain obscure.

THE GOAL

This study was carried out to evaluate the frequency of HHV-6 and HHV-7 infection association with clinical findings in CFS/ME patients in Latvia.

MATERIALS AND METHODS

One hundred and eight patients (37; 34.0% males and 71; 66.0% female, mean age 37 years) with clinically diagnosed CFS/ME corresponding to CDC diagnostic criteria were included in this study. Plasma samples were tested for the presence of HHV-6 IgM and IgG class antibodies using enzyme-linked immunosorbent assay (ELISA) according to the manufacturers' instructions. Deoxyribonucleic acid (DNA) from peripheral blood leukocytes (PBL) and cell free blood plasma was isolated using the phenol-chloroform method. To assure the quality of the PBL DNA and to exclude a possible contamination of plasma by cellular DNA a β-globin gene polymerase chain reaction (PCR) was carried out. Nested polymerase chain reaction (nPCR) was performed to determine the presence of HHV-6 and HHV-7 genomic sequences in DNA isolated from PBL (marker for latent/persistent infection) and from cell free blood plasma (marker for active infection). The obtained amplicones were analyzed by agarose gel electrophoresis. Viral load of HHV-6 in PBL was determined by real time PCR.

RESULTS

HHV-6 specific IgM and IgG class antibodies were detected in 16 (14.8%) and 88 (81.5%) patients respectively, while four (3.7%) had neither IgM nor IgG class antibodies. HHV-6 and/or HHV-7 specific genomic sequences were detected in 91 out of 108 (84.3%) CFS/ME patients' DNA samples: 32 (29.6%) in PBL DNA and 59 (54.6%) plasma and PBL DNA. Virus genomic sequences were not found in 17 out of 108 (15.7%) patients with CFS/ME. Out of 108 patients with CFS/ME, latent/persistent HHV-6 infection was detected in 4 (3.7%) and active HHV-6 infection – in 2 (1.9%) patients. Latent/persistent HHV-7 infection was found in 59 (54.6%) and active HHV-7 infection – in 43 (39.8%) patients, but latent/persistent double HHV-6+HHV-7 infection was found in 28 (25.9%) and active double HHV-6+HHV-7 infection – in 14 (13.0%) patients (Figure 1).

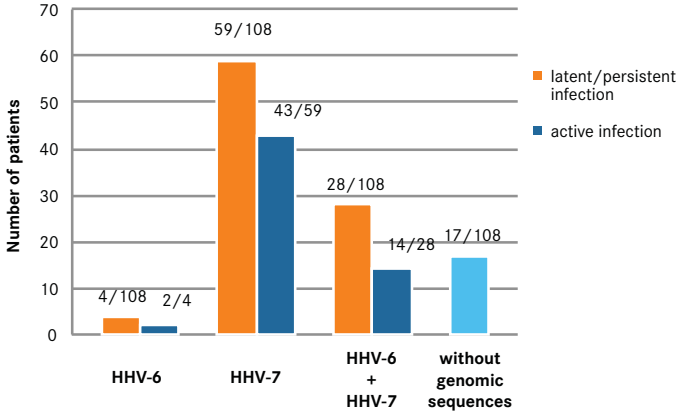

Figure 1. Latent/persistent and active infection of HHV-6 or HHV-7, and concurrent (HHV-6+HHV-7) infection in the patients with chronic fatigue syndrome/myalgic encephalomyelitis

HHV-6 PBL load was higher in the patients with active HHV-6 and HHV-7 co-infection (1007.8± 367.1x10^3 copies/μg DNA) than in the patients with single HHV-6 infection (133.0±10.3x10^3 copies/μg DNA).

Characteristic clinical symptoms of CFS/ME were more often observed in the patients with active HHV-6 and/or HHV-7 infection. Severe chronic fatigue for at least six months or longer was recognized in all patients independently from the cause of the active infection. Subfebrility, tender cervical or axillary lymph nodes and post-exertional malaise were found in the patients with single HHV-7 infection (56.0%, 72.0% and 88.4%, respectively) and HHV-6+HHV-7 co-infection (57.0%, 71.4% and 100.0%, respectively). Muscle pain and muscular asthenia with strong manifestations were detected in all patients. Multi-joint pain was also traced found in all patients with more serious symptoms in co-infection cases. Neuropsychological disorders were detected in all patients: impaired memory – in 85.0% patients with active HHV-7 and HHV-6+HHV-7 infection, and impaired concentration – in all patients with HHV-6 infection, 51.2% of patients with HHV-7 and 57.1% in patients with HHV-6+HHV-7 infection. Un-refreshing sleep was revealed in all patients with sleepiness, more characteristic in patients with HHV-7 (48.8%) and HHV-6+HHV-7 (100.0%) infection, and sleeplessness – in all patients with HHV-6 infection and in 51.2% patients with HHV-7 infection. Headaches of new type were reported from 44.2% patients with HHV-7 infection.

DISCUSSION

Studies addressing the possible involvement of HHV-6 and HHV-7 in CFS/ME have discrepant results. Majority of researchers have reported on an association between HHV-6, HHV-7 and CFS/ME (Chapenko, et al., 2006). HHV-6 DNA was detected in 29.0% of healthy individuals and in 44.0% patients with CFS/ME (Di Luca, et al., 1995). Active HHV-6 infection using HHV-6 specific monoclonal antibodies and PCR is detected in 70% of CFS/ME patients and 20% of healthy blood donors (Krueger & Ablashi 2006). Likewise, Nicolson reports on an association of active HHV-6 infection with CFS/ME (Nicolson, et al., 2003). However, several researchers find no differences in the prevalence of HHV-6 and HHV-7 between CFS/ME patients and healthy controls (Wallace, et al., 1999; Koelle, et al., 2002). Similarly, recent serological and virological investigations detected no differences in HHV-6 prevalence between the control group and CFS/ME patients, however, the groups of the examined persons were rather small which cannot give grounds for drawing general conclusions (Cameron, et al., 2010). In this study HHV-6 specific IgM and IgG class antibodies were detected in 14.8% and 81.5% CFS/ME patients respectively. Comparing to other studies, where elevated titres of HHV-6 IgM class antibodies were detected in 57.1% of CFS/ME patients versus 16.0% in healthy controls (Ablashi, et al., 2000), our study found no differences between the prevalence of HHV-6 specific IgM and IgG class antibodies.

According to our previous study, latent/persistent infection was detected in 53.3% of healthy blood donors: HHV-6 in 3.3%, HHV-7 in 43.3% and dual (HHV-6+HHV-7) in 6.6%. Active HHV-7 infection was found in 10.6% of the examined donors but active HHV-6 infection in none of them (Kozireva, et al., 2001). No statistical differences between the frequency of latent/persistent single HHV-6 and single HHV-7 infection were detected in the CFS/ME patients and the healthy blood donors ($p=1.0000$ and $p=0.3064$, respectively). However, the prevalence of latent/persistent dual (HHV-6+HHV-7) infection was significantly higher in the CFS/ME cases than in the donors ($p=0.0244$). Active HHV-7 infection is detected more often in CFS/ME patients than in healthy blood donors. Active single HHV-6 and active dual (HHV-6+HHV-7) infection was found only in the patients with CFS/ME, thus confirming the involvement of active HHV-6, HHV-7 and dual (HHV-6+HHV-7) infection in the development of CFS/ME. High load of HHV-6 DNA was detected in 15-30% CFS/ME patients (Krueger, et al., 2001; Fremont, et al., 2009). In this study we found higher HHV-6 PBL load in patients with active HHV-6 and HHV-7 co-infection than in patients with single HHV-6 infection, suggesting of a more important role of dual infection in CFS/ME. Severe clinical course of disease (chronic fatigue for at least six months or longer, muscle pain and muscular asthenia, multi-joint pain and un-refreshing sleep) was observed in the patients with active HHV-6 and/or HHV-7 infection. Despite the efforts on developing standardized research criteria for defining CFS/ME, progress in diagnosis and treatment is slow because of lack of a single standard clinical definition and specific biomarkers of the disease (Albright, et al., 2011).

CONCLUSIONS

The high rate of active HHV-7 and concurrent HHV-6+HHV-7 infection suggests that these immunomodulating pathogens could be a trigger factor for CFS/ME development. The association between an active viral infection and severe clinical symptoms claims the need of concurrent study of these viral infections for defining the possible subsets of CFS/ME.

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ASSESSMENT OF HHV-6 AND HHV-7 IN PATIENTS AFTER KIDNEY TRANSPLANTATION: IMPACT ON CLINICAL AND IMMUNE PARAMETERS

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INTRODUCTION

Human herpesvirus (HHV)-6 and -7 belong to the *Betaherpesvirinae* subfamily and are closely related to another member of the subfamily, cytomegalovirus (CMV). Infection with HHV-6 and HHV-7 commonly occurs in childhood and then subsequently results in lifelong latency, making the seroprevalence rate in adults over 90% (1).

Primary HHV-6 infection has been associated with febrile illness, including exanthema subitum and infection, most likely transmitted by means of saliva, usually acquired during the first 2 years of life. The virus uses CD46 as a cellular receptor and therefore HHV-6 may also infect other cell types, such as monocytes, epithelial and endothelial cells (2). The clinical course of primary HHV-6 infection is generally benign and self-limited. However, several severe complications have been reported, including encephalitis/encephalopathy, hepatitis and thrombocytopenia (3). Infection with HHV-7 is also widespread in the population, with primary infection occurring early in life, probably through salivary transmission. HHV-7 has also been associated with febrile illness in children and is another etiologic agent of exanthema subitum (4). The virus uses CD4 as a cellular receptor to infect T cells (5).

While primary infection is uncommon in adult solid organ transplant recipients, reactivation of endogenous latent viruses seems to occur very frequently with infection rates of 30–50% as reported in different studies (6-7). The stimulus for reactivation of beta-herpesviruses is an immunosuppressive (IS) regimen in renal transplant (RT) recipients; however, these viruses possess immunomodulating properties, including the ability to alter the expression of immune activation molecules, modulate expression of several cytokines and chemokines and induce apoptosis in lymphocytes, which may contribute to immunosuppression. Productive infection of CD4 T cells results in cytopathic effects and cell destruction (8). It has been suggested that the HHV-6 infection and activation result in clinical symptoms, including fever, skin rash,

interstitial pneumonitis, bone marrow suppression, encephalitis, and rejection (9). In contrast to the studies of HHV-6 infection in organ-transplant recipients, the number of studies examining HHV-7 infection in these patients is limited. According to several recent studies, HHV-7 may act as a co-factor for CMV activation and CMV disease development in organ-transplant recipients (10).

A growing body of evidence suggests that the major impact of HHV-6 and HHV-7 reactivation in transplantation is related to indirect immunomodulatory effects, such as their association with CMV disease, increased opportunistic infections, and graft dysfunction and rejection (11-12).

In the present study we analyzed the prevalence of latent and active HHV-6 and HHV-7 infection and the impact of this infection on clinical and immunological parameters of graft outcome in renal transplant recipients.

PATIENTS AND METHODS

Fifty recipients transplanted at the Latvian renal transplantation centre between January 2007 and December 2007 and 27 deceased renal allograft donors were included in this prospective study. The mean follow-up was 14 ± 2.5 months. Four early graft losses (until 3 months) occurred because of arterial graft thrombosis (n=1) and death (n=3). The causes of death were surgical peritonitis, pulmonary embolism, but in one case the cause was unknown, because the patient missed a follow-up. Consequently, further clinical, virological data and immunological tests could be obtained from 46 of the 50 patients during a 12 month period. EDTA blood samples were collected from the patients before transplantation, after 2 weeks, 3, 6 and 12 months after the transplantation.

Nested polymerase chain reaction (nPCR) was used for the detection of viral genomic sequences in DNA isolated from peripheral blood leukocytes (PBL) and plasma (markers of latent/persistent and active infection, respectively). DNA was isolated from the whole blood by using the phenol-chloroform method. QIAamp DNA Blood Mini Kit was used to extract DNA from plasma. β-globin PCR was applied in order to check the DNA quality. The corresponding primer pairs were used for the detection of CMV, HHV-6 and HHV-7 genomic sequences (13-15) in DNA isolated from the whole blood and plasma. To exclude the possibility of contamination during the PCR, HHV-6 and HHV-7 negative DNA, as well as water controls were included in each experiment. The amplification products were visualized in 1.7% agaroze gel with ethidium bromide staining and analyzed using Kodak Electrophoresis Documentation and Analysis System (EDAS) 290.

Restriction endonuclease analysis was carried out using enzyme *Hind* III for the detection HHV-6 virus variants (13). This enzyme cuts HHV-6B 163kbp amplimer into two fragments: 66 and 97kbp and does not cut HHV-6A amplimer. This difference shows the distinction between HHV-6A and HHV-6B virus variants.

The concentration of lymphocyte subsets (CD3+, CD4+, CD8+, CD4+/CD8+, CD19+ and CD25+) were measured by flow cytometer (Becton&Dickinson). The number of each cell subsets were expressed as absolute counts of total lymphocytes in 1 mm^3 of peripheral blood.

Clinical parameters (immunosuppressive regimens, acute rejection episodes, transplant function and late complications) were obtained through medical records.

Initial immunosuppression in all patients consisted of cyclosporine (CsA), mycophenolate mofetil (MMF) and prednisolone (P) and induction with monoclonal anti-CD25 antibodies (Basilixibam/Daclizumab). Maintenance immunosuppression consisted of CsA, MMF and P. A treatment with oral CsA in microemulsion was started before surgery (10 mg/kg/d) to obtain therapeutic CsA blood levels and later was adjusted, based on target through levels 150–250 ng/ml during the first four weeks and at 150–200 ng/ml thereafter. The maintenance dose of orally administered MMF was 1.0–2.0 g/d. Methylprednisolone 5.0 mg/kg/d was administered on three consecutive days from the day of RT. Oral prednisolone was started on the first day after operation at 0.5 mg/kg/d and reduced gradually till 5.0–10 mg/d.

Acute rejection (AR) episodes were diagnosed on the basis of characteristic clinical features and were confirmed by percutaneous biopsy. The histological features were graded according to Banff 2005 classification. AR episodes were

treated with intravenous methylprednisolone at 500 mg/d for three consecutive days. Steroid resistant cases were treated with anti-thymocyte globulin (ATG) at 4 mg/kg for 7–10 days.

STATISTICAL ANALYSIS

The data were expressed as mean and standard deviation. Statistical differences between the groups were assessed by the analysis of variance (ANOVA) or Chi-square and *t*-test. A *P*-value < 0.05 was considered statistically significant.

The study was performed with the permission of the Local Ethics Committee and all the participants gave informed consent before the examination.

RESULTS

Latent/persistent ß-herpesviruses infection was detected in 23 out of 27 (85%) transplant donors. Among them CMV infection was found in 7 (26%), HHV-6 – in 5 (18.5%) and HHV-7 infection – in 19 (70%) donors. Active ß-herpesviruses infection was traced found (?) in 4 (15%) donors and from them 2 (7.5%) donors had active CMV and 2 – active HHV-7 infection.

According to the data of ß-herpesviruses genomic sequences detection in PBL DNA samples of the 50 recipients before RT, latent/persistent CMV infection was revealed in 5/50 (10%) recipients, HHV-6 – in 10/50 (20%) and HHV-7 infection – in 39/50 (78%) recipients. At 3 months after RT active ß-herpesviruses infection (*plasma viremia*) was detected in 27 out of 46 (58.7%) recipients. It made 12 (26%) for CMV, 4 (8.6%) for HHV-6, 9 (19.5%) for HHV-7 and 2 (4.3%) for dual (HHV-6 and HHV-7) infection. To analyze the impact of active HHV-6 and HHV-7 infection on clinical and immunological parameters of graft outcome, the recipients were divided into 3 groups: Group I – with active HHV-6 infection (n=4); Group II – with active HHV-7 infection (n=9) and Group III (control) – without active infection (n=31). Two patients with dual (HHV-6 and HHV-7) active ß-herpesviruses infection were excluded due to their small number.

Demografic and clinical results of the recipients with and without active ß-herpesviruses infection are given in Table 1.

Table 1. Main demographic and clinical characteristics of the patients included in the study.

		HHV-6	HHV-7	Control	P
Patients (n)		4	9	31	
Recipient age (years)		41.7±8.1	43.8±13.9	45.7±14.5	0.927
Number of retransplants		0	1 (11.1%)	6 (19.3%)	*0.281; **0.437
Donor age (years)		46.4±12.1	47.2±8.3	46.2±10.2	0.982
ATG induction therapy		0	0	3 (9.6%)	*0.378
Prophylaxis with valganciclovir		3 (75%)	6 (66.6%)	18 (58.0%)	
Acute CMV infection incidence (at 3 months)		0	1 (11.1%)	9 (29%)	*0.266; **0.286
Acute rejection incidence		2 (50%)	3 (33.3%)	10 (32.2)	*0.425; **0.624
Graft function (S-Cr, mmol/L)	At 3 months	0.137±0.042	0.118±0.024	0.125±0.033	*0.515; **0.599
	At 12 months	0.140±0.045	0.118±0.025	0.138±0.056	*0.965; **0.315

**P* controls *versus* group I; ***P* controls *versus* group II

Three patient groups showed no significant differences in the demographic data of the study population. The incidence of acute CMV infection and AR did not differ between the patients who developed active ß-herpesviruses infection and those who did not. It is interesting to note that the patients without active ß-herpesviruses infection had a higher rate of acute CMV infection (29%) compared to patients in the group with active HHV-6 (0%) and HHV-7 (11.1%), respectively. Compared with the controls (32%), AR rate was slightly higher in the patients with active HHV-6 infection (50%), but again, without statistical significance. There was no difference in graft function at 3 and 12 months among the groups as well. The mean serum creatinine at 12 months was even lower in the patients with active HHV-7 compared to the controls (0.11 *vs* 0.13, *p*=0.31).

Table 2 illustrates the lymphocyte subsets number in the peripheral blood of the control group patients with latent/persistent infection compared with the number of cells detected in the patients with active HHV-6 and HHV-7 infection at different time points after RT.

Table 2. Lymphocyte subsets number in the recipients with latent/persistent and active ß-herpesviruses infection.

	Control	HHV-6	P controls vs HHV-6	HHV-7	P controls vs HHV-7
Cell subset before RT					
CD3+	0.650±0.749	0.800±0.589	0.715	0.487±0.372	0.533
CD4+	0.37±0.43	0.45 ±0.38	0.723	0.27±0.25	0.520
CD8+	0.27±0.32	0.41±0.36	0.535	0.21±0.17	0.593
CD4+/CD8+	1.468±0.468	1.167±0.569	0.678	1.443±0.732	0.917
CD19+	0.092±0.117	0.200±0.133	0.210	0.092±0.085	0.883
CD25+	0.044±0.068	0.048±0.038	0.366	0.013±0.013	0.182
Cell subset at 3 months after RT					
CD3+	0.734±0.685	0.842±0.506	0.301	1.149±0.735	0.131
CD4+	0.373±0.403	0.485±0.293	0.331	0.591±0.370	0.155
CD8+	0.322±0.325	0.370±0.228	0.252	0.541±0.431	0.107
CD4+/CD8+	1.335±0.818	1.325±0.419	0.954	1.244±0.665	0.768
CD19+	0.062±0.059	0.070±0.063	0.139	0.111±0.071	0.049
CD25+	0.014±0.016	0.011±0.010	0.064	0.031±0.034	0.030

The lymphocyte subsets analysis before RT revealed a considerably decreased level of CD3+; CD4+; CD8+ cells, but no significant changes among the three groups were found. There were no significant changes in CD4+/CD8+; CD19+ and CD25+ counts in the study groups as well. At 3 months after RT, increment of CD3+ and CD4+ cell levels was observed only in HHV-7 group comparing to the control group (1.14 *vs* 0.73, *P*=0.1 and 0.59 *vs* 0.37, p=0.1, respectively). Compared with the control group, HHV-7 group associated with significantly increased CD19+ and CD25+ cells (*P*=0.049 and *P*=0.03, respectively).

DISCUSSION

The drawback of our study was small study groups. Due to that we could not reach statistical significance in many parameters. Consistent with our previous studies, we found a high incidence of latent/persistent CMV, HHV-6 and HHV-7 infection in the renal donors and the recipients as well (26%; 18.5%; 70% and 10%; 20% and 78%,

respectively). Differing from our previous studies, in this study a lower overall rate of active CMV, HHV-6 and HHV-7 infection was found (26%; 19.5% and 4.3%, respectively). This finding may be due to more intensive prophylaxis with valganciclovir in the latest years. We did not find any significant association between active ß-herpesviruses infection and AR and graft function in recipients at different time points after RT. We revealed a considerably depressed CD3+; CD4+ and CD8+ cell count before RT. This finding characterizes impaired cellular immune function in patients with the end-stage renal failure. Secondary immune failure in uremia is multi-faceted and is influenced by uremic intoxication *per se* by altered renal metabolism of immunologically active proteins and by specific effects of renal replacement therapy (16). It is interesting that CD19+ and CD25+ cell count was significantly increased in HHV-7 group compared with the control 3 months after RT (P=0.04 and P=0.03, respectively). It is possible to speculate that the increase in the expression of CD19+ and CD25+ cells in HHV-7 group could be caused by down-regulation of cellular and humoral immune response due to viral indirect immunomodulatory effects (5, 8, 11). On the other hand, a normal or slightly decreased overall lymphocyte subsets count and a relatively low rate of complications after RT show that maintenance IS regimen with CsA, MMF and P does not induce overimmunosuppression in patients after RT.

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RELATIONSHIP BETWEEN BETA-HERPESVIRUSES REACTIVATION AND COMPLICATIONS DEVELOPMENT AFTER PERIPHERAL BLOOD STEM CELL TRANSPLANTATION

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INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is an important method of standard approach in the treatment of hematological malignancies. The stem cells harvested from peripheral blood (PBSC) are now used with increasing frequency for both autologous (auto-PBSCT) and allogeneic (allo-PBSCT) transplantation (1, 2). Usage of different transplant type has been determined by clinical diagnose of a disease. Auto-PBSC transplantation is used to treat Hodgkin's lymphoma, non-Hodgkin's lymphoma, myeloma and allo-PBSCT – to treat different acute forms of leukaemia (3, 4). Despite successful HSCT, the viral infections remain one of the causes of post-transplant morbidity and mortality.

Human herpesvirus-6 (HHV-6) and -7 (HHV-7) belong to the family *Herpesviridae*, the subfamily *Betaherpesvirinae*, the genus *Roseolovirus* (5, 6). Both viruses are ubiquitous (seroprevalence rate in adults 50–90%) as T-lymphotropic immunomodulating viruses after primary infection establish a lifetime latent/persistent infection and can be reactivated in immunocompromised and immunosuppressed hosts (7, 8). The viruses display primary tropism to CD4+ T cells and CD34+ cells, which are a major source of hematopoietic progenitor cells for transplantation (9, 10). Two distinct variants of HHV-6 (A and B) have been described (11). HHV-6B infection reactivation is a common event in bone marrow and allo-HSC transplant recipients, most likely through reactivation of recipient's virus or re-infection (12), and has been much more frequently identified as the cause of complications development in transplant patients than HHV-6A (13–15). Activation of HHV-6 infection in the early period after transplantation is associated with the range of clinical symptoms (fever, coetaneous rash, acute graft versus host disease [GVHD]). A unique form of HHV-6 persistence is the integration of viral DNA into host chromosomes. HHV-6 integration is characterized by high viral load in whole blood and sera that required the differentiation between primary infection, viruses reactivation and viral integration (16). Although HHV-6 is considered an important opportunistic and potentially fatal pathogen for HSCT patients, the clinical significance of the virus infection remains controversial. The pathogenic role of HHV-7 still remains unclear as well as the interaction between this virus and temporal activation of HHV-6 infection. However, application of different antiviral specific drugs for the prevention and treatment of beta-herpesviruses activation remains contradictory (17).

The aim of this study was to evaluate the incidence of latent/persistent HHV-6 and HHV-7 infection and the activation frequency of these viruses infection in an early period after auto- and related allo-PBSCT; the potential interaction with post-transplant complication development; the potential interactions between both viruses.

PATIENTS AND METHODS

In this retrospective study 44 patients (27 females, 17 males; mean age 34.1±10.7; group I) who underwent auto- and 12 patients who underwent related allo-PBSCT (6 females, 6 males; mean age 35.7±7.2; group II) were involved. Group I was composed of 29 patients with Hodgkin's disease, 5 with non-Hodgkin's lymphoma, and 10 patients with myeloma. From 12 patients with allo-PBSC transplantation from related donors in 7 patients – acute myeloid leukaemia, in 3 – acute lymphoblastic leukaemia and in 2 patients – Hodgkin's disease was diagnosed. Pre-transplant myeloablative therapy for lymphoma patients was carried out by BEAM, for myeloma patients – with melphalan and a variety of protocols were used for other diagnoses. To limit the risks of GVHD allo-PBSCT recipients received immunosuppressive therapies (cyclosporine or medrole). For prophylaxis against herpesviruses activation and bacterial infections valacyclovir and trimethoprinum after transplantation were administered to all patients. The cohort was established with the approval of the Ethics Committee of Rīga Stradiņš University and all participants gave their informed consent prior to the examination. EDTA blood samples were collected from the patients before transplantation as baseline and during 3 months after transplantation.

HHV-6 serology

HHV-6 specific IgM and IgG class antibodies were detected using ELISA kits (Panbio, Australia) according to the manufacturer's recommendations.

Viral DNA detection

Nested polymerase chain reaction (nPCR) was used for the detection of HHV-6 and HHV-7 genomic sequences in DNA isolated from peripheral blood leukocytes (PBL) and cell free plasma (markers of latent/persistent and active infection, respectively). Positive (virus genomic DNA) and negative (DNA without virus-specific sequences) as well as water controls were included in each experiment. HHV-6 viral load in PBL DNS samples was detected using HHV-6 Real-Time Alert Q-PCR kit (Nanogen, USA) and Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, USA).

Restriction endonuclease analysis was carried out using enzyme *Hind* III for the detection HHV-6A and HHV-6B virus variants.

Assays for cytokine determinations

ELISA kits (Pierce Biotechnology, USA, and Biosource, Belgium) were used for TNF-α, IL-1β, IL-6, IL-2 and IL-4 levels in serum/plasma samples.

sIL-2R level was measured using Solid-phase competitive chemiluminiscent enzyme immunoassay (CLIA, IMMULITE Operators Manual, 2002, Version 4xx).

STATISTICAL METHODS

Statistical difference in the prevalence of latent/persistent and active HHV-6 and HHV-7 infection was assessed by Fisher's exact test. SPSS software was used to assess the continuous variable values of cytokines levels with a value of $p<0.05$ considered as significant.

RESULTS

HHV-6 serology

Significant difference in the prevalence of anti-HHV-6 specific IgG class antibodies was not detected between auto- and allo-PBSCT patients before transplantation (36/44, 81.8% and 10/12, 83.3%, respectively). Simultaneous presence of IgM and IgG class antibodies was found in 2.3% (1/44) of group I patients and in 16.7% (2/12, $p=0.11$) of group II patients. Anti-HHV-6 IgG class antibodies only were revealed in 83.3% (10/12) donors.

Viral DNA detection in PBL and plasma samples before PBSCT

Beta-herpesviruses genomic sequences were found in 86.4% (38/44) DNA PBL samples from group I and in 100% samples (12/12) from group II pre-transplant patients (Table 1).

Table 1. Detection of latent and active beta-herpesviruses infection in auto- and allo-PBSCT patients (nPCR)

Viral infection	Before auto-PBSCT (group I, n=44)		Before allo-PBSCT (group II, n=12)		After auto-PBSCT (group I)	After allo-PBSCT (group II)
	PBL	Plasma	PBL	Plasma	Plasma	Plasma
Single HHV-6	2/44	0/2	0/12	0/12	0/2	0/12
Single HHV-7	20/44	4/20	7/12	2/7	9/20	4/7
HHV-6+HHV-7	16/44	1/16	5/12	1/5	9/16	3/5
Negative	6/44	0/6	0/12	0/12	0/6	0/12

From 44 patients of group I latent/persistent single HHV-6 infection was detected in 4.5% (2/44) of the patients, single HHV-7 infection – in 45.5% (20/44) and concurrent HHV-6+HHV-7 infection – in 36.4% (16/44) of the patients. From 12 patients of group II single HHV-6 infection was not revealed, single HHV-7 infection was found in 58.3% (7/12) and dual infection – in 41.7% (5/12) of the patients. Latent/persistent viral infection was found in 75% (9/12) of the transplant donors (single HHV-7 in 7/12 and concurrent HHV-6+HHV-7 infection – in 2/12). In all 23 PBL DNA samples from the patients and in 2 samples from the donors with HHV-6 infection HHV-6B variant was identified.

Plasma viremia (PV) was found in 13.2% (5/38) of the auto-PBSCT patients. Out of them HHV-7 PV was detected in 4 patients with single HHV-7 infection and in one patient – simultaneous HHV-6+HHV-7 PV. Active viral infection was detected in 25.0% (3/12) allo-PBSCT patients, out of them active HHV-7 – in 2 patients with single infection and in one patient active HHV-6+HHV-7 infection. No significant difference in the rate of PV between group I and group II patients was revealed. Active HHV-7 infection was found in one transplant donor.

PRESENCE OF ACTIVE BETA-HERPESVIRUSES INFECTION AFTER PBSCT

The frequency of PV in the auto-PBST patients (18/38, 47.4%) as well as simultaneous HHV-6+HHV-7 infection reactivation (9/38, 23.7%) were significantly higher after transplantation in comparison with the frequency before transplantation (p=0.0003, p=0.014, respectively) (Table 1). Increase at twice the rate of active viral infections after transplantation in the allo-transplant patients (7/12, 58.3%; HHV-7 in four and simultaneous HHV-6+HHV-7 in three) was revealed. The increase of mean HHV-6 load was detected in PBL DNA of the patients examined in the period of the active phase infection in comparison with the load in the period of the latent phase infection (54676 and 4781 copies/μg DNA, respectively).

HHV-6B genomic sequence was detected in all 14 plasma DNA samples from the patients with HHV-6 PV before and/or after transplantation.

The mean number of days before detecting HHV-7 and HHV-6 reactivation was 11 days (range: 8 -14 days) and 27 days (range: 12–34 days), respectively.

PLASMA/SERUM EXPRESSION LEVELS OF CYTOKINES

The measure of plasma/serum pro-inflammatory (TNF-α, IL-6, IL-1β), IL-2, IL-4 and sIL-2R cytokine levels showed a significant increase in mean expression levels of TNF-α, IL-6, IL-1β and sIL-2R in the both patient groups with active viral infection in comparison to the levels in the group of patients with latent infection ($p<0.0001$, $p<0.035$, $p<0.0001$, $p<0.0004$, respectively) (Table 2). None of the transplant patients had detectable value of IL-4 and IL-2 in the serums/plasma samples.

Table 2. Plasma/serum expression levels of cytokines in the patients with PBSCT

Viral infection	TNF-alpha (pg/ml)	IL-6 (pg/ml)	IL1-beta (pg/ml)	IL-4 (pg/ml)	IL-2 (pg/ml)	sIL-2R (U/ml)
Latent	11.69±3.12	5.28±1.30	21.86±7.26	<2	<2	987.0 ±341.15
Active	59.88±7.75	13.23±2.19	83.45±12.39	<2	<2	2228.0±736.34

Relationship between active beta-herpesviruses infection and the clinical complications development in an early period after PBSCT

Different clinical complications not related to the basic disease were detected in 12 out of 44 (27.3%) auto-PBSC transplant patients and activation of the one (4/12) or both viruses (8/12) was found in all them. In 8 out of 12 patients febrile syndrome was diagnosed (3–14 days) frequently preceded or combined with gastroenteritis and/or pneumonitis (4/8 patients) development. Complications after allo-PBSCT that may be associated with viral infection activation were diagnosed in 5 patients: febrile syndrome – in 4 and febrile syndrome, cutaneous rash and acute GVHD – in one patient.

DISCUSSION

Our data show high frequency of detectable HHV-6 and HHV-7 latent/persistent infection in stem cell transplant patients in Latvia that makes it possible to determine the risk of these viruses infection activation after auto- and allo-PBSCT and the validity of antiviral prophylaxis and pre-emptive therapy application.

The detection frequency of active HHV-6 and HHV-7 infection considerably increases after auto- as well as allo-PBSCT (45.0% and 58.3%, respectively) compared to that before transplantation (12.5% and 13.2%, respectively). The rate of single HHV-7 and dual (HHV-6+HHV-7) infection activation is prevalent in the both groups of patients. The presence of active viral infection before PBSCT can be a consequence of the application of pre-transplant chemotherapy in the patients of both groups. Moreover, the high rate of active viral infection in the patients after allo-PBSCT can be associated with immunosuppression provoked by using of immunosuppressive treatment (cyclosporine or medrole). Our data and experience suggest that valacyclovir used for antiviral prophylaxis is not sufficiently effective to prevent HHV-6 and HHV-7 infection activation as it had been observed preveiously by other researches (17).

Previous investigations have shown that *Roseoloviruses* are effective modulators of immune response, mainly by modulating the production of pro-infammatory cytokines (8). Our results also show significantly higher expression levels of TNF-α, IL-1β, IL-6 and sIL-2R in plasma/serum samples from the patients with active viral infections.

At present the issue of potential interaction between HHV-6 and HHV-7 is not clear (18–19). The data of this study reveal activation of HHV-7 before HHV-6 activation in the patients with dual (HHV-6+HHV-7) infection that may suggest interaction between these both *Roseoloviruses*. Elucidation of the mechanisms explaining the relationship between these viruses awaits further study.

The relationship between active HHV-6 and HHV-7 infection and clinical episodes in an early period after PBSCT is not clear. In this study different clinical complications are diagnosed in 27.3% (12/44) auto-PBST patients and in 41.7% allo-PBSCT patients (5/12) and in all of them active beta-herpesviruses infection is detected, suggesting causal relationship between these processes. In the present study an interesting finding is fever development, appearing of skin rash and acute GVHD in the allo-PBSCT patient with previous HHV-6 infection activation suggesting the role of HHV-6 as a trigger for GVHD. Analogous symptoms are observed by Yoshikawa, et al. (20) and Pichereau, et al. (21) in bone marrow transplant recipients.

Transmission of HHV-6 from the donor with the allograft has been documented and mononuclear cells latently infected with HHV-6 in the donor allograft are believed to be the likely source of transmission (22). Such a possibility might be proposed as in one patient who did not have the antibodies to HHV-6 before transplantation and received the transplant from HHV-6 positive donor HHV-6 infection after transplantation was diagnosed.

CONCLUSION

High frequency of HHV-6 and HHV-7 infection activation with a simultaneous increase of pro-inflammatory cytokines plasma/serum levels suggests that both viruses are involved in the complication development in the early period after auto- and allo-PBSCT via their immunomodulatory ability. The kinetics of the virus activation reflects the potential role of HHV-7 as a co-factor of HHV-6 activation.

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PRESENCE OF HHV-6A, HHV-6B AND EBV DNA IN PERIPHERAL BLOOD OF PRIMARY CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS AND ITS INFLUENCE ON B-CELL SUBPOPULATION PROFILE

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INTRODUCTION

Human herpesvirus 6 (HHV-6) was first reported in 1986, as human B-lymphotropic virus. The name was subsequently changed to human herpesvirus 6 (Salahuddin, et al., 1986) and it was identified as a member of the β family of herpesviruses (Lusso, et al. 1995). Seroepidemiological surveys reveal that antibodies to HHV-6 are highly prevalent in human populations in different geographical areas; the prevalence varying between 70–100% (De Bolle, et al., 2005). Clinical presentations of HHV-6 infection differ depending upon the age and immune competence of the person. In immunocompromised hosts the spectrum of specific EBV and HHV-6 clinical syndromes are associated with a worse outcome (Singh, et al., 2004). They both have an ability to cause latent infection with reactivation during periods of immunosuppression (Tailor, et al., 2004). Activation of HHV-6 was seen in CLL as a result of lymphoma associated immunosuppression (Loutfy, et al., 2010). Multiple studies have suggested an association between both HHV-6 and EBV and pathogenesis of lymphoma (Diepstra, et al., 2005).

A unique form of HHV-6 persistence is characterized by integration of the viral DNA sequences into chromosomes. The incidence of chromosomal integration for HHV-6 is about 2% in the population of the United Kingdom. Integration of HHV-6 genome was demonstrated in Burkitts lymphoma cell line (Daibata, et al., 1998). These findings suggest the possibility of an association between chromosomally integrated HHV-6 and the development of haematological malignancies. However, transforming events after HHV-6 infection have not been confirmed in vitro, and no definite association between HHV-6 and malignant transformation have been proved in vivo (Ogata, 2009). EBV is well-known as oncogenic agent. EBV has been shown to represent an oncogenic infectious agent for Hodgkin's lymphoma and was present in the neoplastic cells in 20–40% of patients with Hodgkin's lymphoma (Diepstra, et al., 2005)[7]. The presence of HHV-6 has been reported in 50–68% of patients with different malignant lymphomas (reviewed in Sumiyoshi, et al., 1993). However, whether HHV-6 or EBV plays a role in lymphatic malignancies like CLL remains unclear.

Chronic lymphocytic leukemia (CLL) is a chronic lymphoproliferative disorder characterized by the accumulation of monoclonal B cells in the blood, bone marrow, and secondary lymphoid tissues (Faguet, et al., 1994). The age-adjusted incidence rate is 4.1 per 100 000 men and women per year. According to the WHO classification (2008) CLL is always a B-cell neoplasm and the diagnosis of CLL requires the detection of a clonal population of small mature (CD34-negative, CD10-negative, CD5-positive) B-lymphocytes in peripheral blood (PB) (Gribben, 2010). The current view treats CLL as a disease characterized by a dynamic balance between cells located and actively proliferating in permissive niches in the lymphoid organs and those circulating in the blood and resisting programmed cell death (Deaglio, et al., 2010).

Traffic of the B-cell subsets between tissues, where development of B-cells takes place, through peripheral blood reflects the immune status of an individual subject and potentially also disorders of B-cell development, autoimmunity, and lymphoproliferative diseases. PB circulating B-cell subsets have been poorly defined until recently, when ≥6-color flow cytometry became routinely available (Caraux, 2010). The analysis of steady-state PB samples from 614 healthy adults by polychromatic flow cytometry (PFC) defined four systematically circulating B-cell subpopulations: immature (mean 5.4%), naïve (mean 64%), memory (mean 31%) and plasma cells (mean 2.1%). The frequency of immature B-lymphocytes in PB has been found to be increased in autoimmune and other immunological diseases (Perez-Andres, et al., 2010).

The aim of our study was to analyze and quantify two B-cell sub-populations, immature CD10+ and mature CD10-, in PB samples from 20 CLL patients in order to find out whether the data correlate with the clinical stage of the disease. At the same time we wanted to verify whether the presence of HHV-6 and/or EBV DNA could influence the PB B-cell subsets at different clinical stages of CLL.

MATERIALS AND METHODS

Peripheral blood samples were collected from 20 patients diagnosed with CLL. Combined Rai-Binet staging system was used to divide the patients into groups. The Binet and Rai systems of staging are used virtually all over the world. The International Workshop on CLL recommended combining these two systems, designating each Binet stage by the corresponding Rai stage in parenthesis (International Workshop on CLL/IWCLL/, 1981). Six patients (30%) had combined Rai-Binet stage IA disease; 4 patients (20%) had stage IIA disease; 6 patients (30%) had stage IIB disease; 4 patients (20%) had stage IIIB disease.

In the present study the DNA isolated from mononuclear peripheral blood cells was subjected to PCR analysis. High molecular weight DNA was prepared by proteinase K digestion and phenol/chloroform extraction according to standard procedures (Sambrook, et al., 1989).

HHV-6 AND EBV DNA DETECTION

Presence of HHV-6 DNA (both strains A and B) was assayed by nested PCR using two sets of primers. This set of primers was derived from a highly conserved region shown to encode the major capsid protein. The sequences of the primers and the PCR protocol were reported by Secchiero, et al., 1995. The sensitivity of the current HHV6 nested PCR was defined by end-point serial dilutions with HHV-6 DNA control (HHV6A GA Quantitated Viral DNA, Advanced Biotechnologies Inc) as 5 copies per reaction (500 ng) or per ~7,6x104 diploid cells. Presence of EBV DNA was assayed by 2-steps PCR using one set of primers from the Cp promoter region of EBV genome: Cp-F (5`-AAGACCTACGCCTCTCCATTCATC-3`) and Cp-R (5`-AGGGGAAGTGACCCCGAAAT-3`), that yields a 362-bp fragment. These primers were designed using Primer3 program (http://wi.mit.edu) and the EBV genes mRNA reference sequences (RefSeq) from public Sequences database at NCBI (USA) (http://www.ncbi.nlm.nih.gov). The primers specificity was verified by running the sequences through the whole human genome sequences databases (NCBI) using the BLAST programme. The 2-steps PCR was carried out using the same protocol as for HHV-6 nested PCR, but with the annealing of Cp-F and Cp-R primers at 60 °C.

FLOW CYTOMETRY

Peripheral blood samples from the CLL patients and the healthy individuals were stained using PerCP-Cy5.5 (the tandem conjugate that combines peridinin-chlorophyll protein with a cyanine dye) or PE-Cy5 (the tandem conjugate that combines phycoerythrin and a cyanine dye) conjugated monoclonal antibodies against CD19 and PE (phycoerythrin) conjugated monoclonal antibodies against CD10 (BD Biosciences, USA). Fluorescence measurements were made by fluorescence-activated cell sorter BD FACS Aria II (BD Biosciences, USA). BD FACS Diva 6.2 software was used to analyze the data. FSC (forward scatter), SSC (side scatter) parameters and fluorescence intensity of CD19 receptor

expression were used to determine B-cell population. Fluorescence intensity of CD10 receptor expression was used to determine B-cell subpopulations. The mean fluorescence intensity (MFI) of CD19 and CD10 receptor expression on the surface of peripheral blood B cells was compared with normal controls.

This study was approved by Rīga Stradiņš University Review Board and was conducted in accordance with the ethical guidelines mandated by the Declaration of Helsinki.

STATISTICAL METHODS

Descriptive statistics were used to analyze the data and summarize baseline characteristics. Median and range or mean and standard deviation (± SD), frequencies and percentages were used, as appropriate. Kruskal-Wallis test was used to compare the data from multiple groups.

RESULTS

The median age of the 20 CLL patients in this study was 74.2 ± 7 years (range 66 – 86 years). Male/female ratio was 3:1.

There was no significant difference in B-cell count and expression of CD19 between the CLL and the control groups. Results of the flow cytometry (FC) analysis suggested that the expression of CD10 receptor on the surface of peripheral B-cells can vary significantly and allows distinguishing several separate subpopulations. Two subpopulations of B-cells were detected in the peripheral blood of the normal donors – CD19+/CD10+dim (immature B-cells, which retained dim expression of CD10 (Van Lochem, et al., 2004) and CD19+/CD10-(naïve, memory and plasmatic B-cells). In our study three subpopulations of B-cells were identified in the peripheral blood of the CLL patients – CD19+/CD10+dim (immature B-cells), CD19+/CD10++bright (abnormal B-cell clones) and CD19+/CD10-(naïve, memory, plasmatic and abnormal B-cells which did not express CD10) (Table 1).

Table 1. Distribution of mature (CD10-negative) and immature (CD10-positive) B-cell in the peripheral blood of the CLL patients, according to the clinical stage of the disease (Mean ± Standard deviation).

CLL patient groups classified according to Rai-Binet combined staging system					Control group (healthy donors)
	IA (n=6)	**IIA (n=4)**	**IIB (n=6)**	**IIIB (n=4)**	**(n=9)**
CD19+	70 ± 17.3	82.4 ± 11.3	66 ± 22.1	75.6 ± 14.5	85 ± 7.2
CD19+/CD10+	3.49 ± 3.3	3.87 ± 1.5	9 ± 13.2	7.09 ± 6.4	11.5 ± 4.9
CD19+/CD10++	57 ± 19.7	24.9 ± 31.4	8.81 ± 14.8	16.2 ± 32.3	-
CD19+/CD10-	36 ± 21.8	66.3 ± 34.6	77 ± 24.8	74.1 ± 32.7	85 ± 7.2

\+ dim population according to FC analysis, ++ bright population according to FC analysis

Kruskal-Wallis test showed a significant difference between the groups; statistic H was 19.01; chi square = 9.4; p = 0.05. Clinical evidence indicated that CD10 expression can alter over time (Hamblin, et al., 2009). We have shown that progression of the disease was accompanied by changes in subpopulation profile (Figure 1). Subpopulations of peripheral B-cells in CLL patients expressed significantly higher levels of CD10, in comparison to normal circulating B-cells.

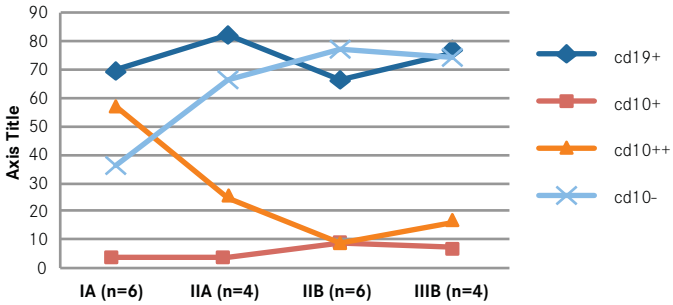

Figure 1. Distribution of mature (CD10-negative) and immature (CD10-positive) B-cells in the peripheral blood of the CLL patients, according to the clinical stage of CLL. There is a marked decrease of CD10++ (bright) B-cell subpopulation and increase of CD10- B-cell subpopulation during progression of the disease.

Mononuclear peripheral blood cells from two patients (10%) were found to be positive for HHV-6 DNA by PCR assay (defined sensitivity – 5 copies per reaction). These patients belonged to different stage groups – IIA and IIIB (Figure 2). By 2-step EBV-Cp PCR (defined sensitivity – 10 copies per reaction) the blood samples from all CLL patients in the study were EBV-negative.

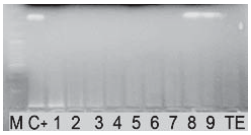

Figure 2. HHV-6 nested PCR analysis of the leukocytes isolated from the blood of the CLL patients. The HHV-6-specific PCR-product is 258-bp in size. 1 – 9, number of the patient; C, HHV-6 DNA control (HHV-6A GA Quantitated Viral DNA, Advanced Biotechnologies Inc) 10 copies per reaction; TE, TE-buffer as a negative control; M, DNA marker; 500 ng of DNA were used for nested PCR.

According to the results of FC analysis of the HHV-6 positive CLL patients, B-cell subpopulation profile differs from that of the patients in the same stage group – there was a decrease in CD19+ and CD19+/CD10- subpopulation count and increase in expression of CD10 (CD19+/CD10+ dim and CD19+/CD10++ bright subpopulation) (Figure 3, 4.).

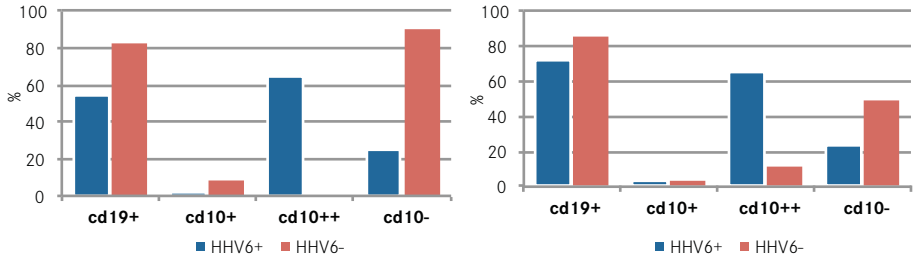

Figure 3 and 4. Changes of B-cell subpopulation profile (expression of B-cell surface receptors) in HHV-6 positive patients. Mature (CD10-) and immature (CD10+ and CD10++) B-cell subpopulations in HHV-6-positive and HHV-6-negative CLL patients.

DISCUSSION

Many studies aimed at identifying HHV-6 genome in pathologic specimens using polymerase chain reaction have been reported. Frequencies of positive HHV-6 DNA appear to vary widely among these studies, and may depend on the differences in PCR sensitivity for each study. It is important to note that positive PCR results do not necessarily indicate the presence of HHV-6 in neoplastic cells: HHV-6 is a ubiquitous pathogen, and may remain in a latent state in various host cells including leukocytes (Singh, et al., 2004). Alterations of immune status due to disease or immunological dysfunction may induce HHV-6 reactivation.

Examinations of HHV-6 antigen expression in tumour tissue would improve the interpretation of results. Up to date, relatively few studies have focused on HHV-6 expression on neoplastic cells, and no evidence has been found for the involvement of HHV-6 in neoplastic cells.

Potential involvement of HHV-6 in pathogenesis of CLL could be due to: (a) immunosuppression from both lymphatic malignancy and its treatment that may predispose patients to a higher risk of co-infection, (b) an immunomodulating effect of HHV-6 since it can induce production of interleukin-1β and tumour necrosis factor-alpha, suppress T lymphocyte function due to reduced interleukin-2 synthesis (Flamand, et al., 1995), and suppress bone marrow (Flamand, et al., 1991) by inducing interferon- alpha production (Tesch, et al., 1993). (c) HHV-6 can directly infect CD4+ T-cells and induce apoptosis, thus altering key immune activation molecular pathways and subsequently disturbing the cytokine network. (d) HHV-6 can also infect thymic epithelial cells, hematopoietic stem cells, and natural killer cells, which are critical for immune maturation and protection against cancer and viral infections.

In the present cross-sectional study a single sample from each patient was analyzed, and the timing, duration and the course of infection could not be established. Quantitative analyses of peripheral blood cells, serum and tissues in follow-up studies are necessary for better understanding of the HHV-6 influence.

CONCLUSIONS

Our results suggest that the profile of two PB B-cell subsets, immature (CD10+) and mature (CD10-) is characteristic of the clinical stages of CLL disease and distinguishes the early stage I from advanced stages. The monitoring of peripheral blood B-cell sub-populations during a follow-up of CLL patients may be proposed as a disease progression indicator.

The presence of HHV-6 could be considered as a predicting indicator of cellular immunosuppression, but further prospective follow-up studies are required to identify a pathogenic role of HHV-6 with each CLL patient.

Although CLL remains incurable, advances in the use of prognostic factors that identify patients at high risk for progression will lead to increased treatment response rates and the duration of response.

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CELLULAR IMMUNE RESPONSE IN HHV-6/-7 INFECTED GASTROINTESTINAL CANCER PATIENTS

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INTRODUCTION

Human herpesvirus-6 (HHV-6) and human herpesvirus-7 (HHV-7) are ubiquitous beta-herpesviruses and remain latent in the host after primary infection and persist lifelong in the body. Nevertheless, these viruses can be reactivated in immunosuppressed conditions leading to critical outcomes such as interstitial pneumonitis and encephalitis [1, 2]. Malignancy is associated with immunosuppression in hematological tumours and in solid organ cancers as well.

Cellular immunity is the most important part of the immune system in anticancer immune response. Numerous innate and adaptive immune effector cells and molecules participate in the recognition and destruction of cancer cells, a process that is known as cancer immune surveillance. However, cancer cells avoid such immune surveillance through the outgrowth of poorly immunogenic tumour cell variants, called immune selection, and through suppression of the immune system [3]. Cancer-associated immunosuppression is mediated by evaluation of an immunosuppressive network that extends from the primary tumour site to secondary lymphoid organs and peripheral blood immunocompetent cells. Chronic stimulation of T cells by tumours leads to activation-induced cell death and insufficiency of cellular immune response. It could be expected that each additional immunosuppressive factor contributes to the tumour escaping from immunological control and promotes cancer growth.

Human herpesviruses (HHV) are frequently present in patients with a compromised immune system and are described as immunotropic viruses that can infect several cells implicated in the generation both cell-mediated and humoral immune response. Beta-herpesviruses HHV-6 and HHV-7 have some similar biological properties and both viruses are often recognized concomitantly suggesting that viral syndromes may often be due to a combination of these viruses [4]. HHV-6 and HHV-7 can induce immunosuppression by various mechanisms and triggering of apoptosis in lymphocytes is one of the most important [5].

CD4+ T lymphocytes appear to be the preferential target for replication of HHV-6 as well as HHV-7 viruses *in vivo*. In addition CD8+ T cells, monocytes/macrophages, natural killer (NK) cells, epithelial, endothelial, neural cells and fibroblasts may be infected [6, 7].

An effective CD4+ T cells response is believed to prevent tolerance induction by tumour antigen, effective function of cytotoxic CD8+ T cells thereby prevent tumour escape from immunological control. T cells help is also involved in the deletion of cytotoxic T-ly in the chronic viral infection. The persisting presence of low levels of antigens in these cases is similar to some cancers. Therefore it is possible that cytotoxic T-ly against tumour antigens is deleted when T helper responses are absent [8].

HHV-6 influence of cytokine secretion is described as one of the possible modes to modulate the host immune system. HHV-6 suppressed secretion of IL-12 by macrophages and dendritic cells [9] as well as IL-2 secretion [10] and induces inflammatory and immunosuppressive cytokines such as IL-1 beta, IL-10, TNF-alpha [11]. Virus-induced changes in cytokines secretion lead to changes in tumour microenvironment and deviation of antitumour immune response.

Beta-herpesviruses association with and influence on chronic fatigue syndrome, solid organ and stem cells transplant recipients, multiple sclerosis is well documented [12, 13]. HHV-6 and HHV-7 are frequently present in patients with various lymphoproliferative disorders, including multiple myeloma, Hodgkin's disease, T-cell lymphoma and myeloproliferative syndromes. Additionally HHV-6 has been associated with precancerous lesions of the uterine cervix [14], while immunomodulating processes between the host and the virus during HHV-6 and HHV-7 infection in solid tumour patients has not been not widely investigated.

Our aim was to clarify the influence of beta-herpesviruses on cellular immune response in gastrointestinal cancer patients at the time of tumour diagnosis.

MATERIALS AND METHODS

We examined 95 gastrointestinal (GI) cancer patients before the antitumour treatment. The patients' age was from 38 to 75 years. The patients were divided into two groups according to lymphocyte count in peripheral blood: Ly > 1400 per 1 mm3 and Ly < 1400 1 mm3 (group I and II, respectively). HHV-6 and HHV-7 were detected in both groups. Lymphocyte subpopulations were determined by the laser flow cytofluorometer Becton Dickinson with corresponding monoclonal antibodies to CD3+, CD4+, CD8+, CD16+, CD19+, CD38+, CD25+, CD95+ lymphocytes. Nested polymerase chain reaction (nPCR) was used for the identification of viral sequences in DNA isolated from peripheral blood leukocytes (PBL) and plasma (markers of latent/persistent and active infection, respectively). The investigation was carried out with the approval of the Ethics Committee of Rīga Stradiņš University and all patients have given their informed consent prior to the examination. As statistical method, Fisher's exact test, NPar, Kruskal Walles test for statistical evolution of the results were used. 27 patients were examined for IL-6, sIL2R and TNF-alpha in serum by hemiluminiscent immunoassay using analyzer Immulite 1000.

RESULTS

The distribution of patients with HHV-6, HHV-7 infection is given in Table 1.

Table 1. HHV-6 or HHV-7 infection in the GI cancer patients.

Infection	HHV-6 n	HHV-6 %	HHV-7 n	HHV-7 %
negative	83	87.4	33	34.7
latent	11	11.6	39	41.1
active	1	1.1	23	24.2

One of the patients had both HHV-6 and HHV-7 active infections.

There was no statistically significant difference between leukocyte, lymphocyte, monocyte and CD3, CD4, CD8, CD38, CD16, CD19, CD95, CD25 cell absolute counts and CD4/CD8 ratio between negative, latent and active HHV6 and HHV7 infection groups in the GI cancer patients (the data have not been presented in Table 1). However, we observed a tendency of increased number of CD3+, CD4+, CD8+, CD38+ and CD95+ cells in peripheral blood in the GI cancer patients with active beta-herpesvirus infection. It was surprising that the total number of CD16+ cells was not influenced by the virus infection in our patients.

Cellular immune parameters were determined in both immunocompetent (Ly > 1,400) and immunocompromised (Ly < 1,400) GI cancer patients groups, irrespectively of beta-herpesvirus infection. The number of T cells and NK cells was higher as expected, in the patients without lymphopenia, while the total count of B cells and CD25+ cells was similar in both groups (Table 2).

Table 2. Absolute count of immunocompetent cells in the GI patients in group I and II

Parameters	Absolute group I: ly >1400	count ± SD group II: ly <1400	p
L	7140±2044	5360±1962	<0.001*
Mo	587±199	460±190	<0.05*
CD3	1545±523	802±209	<0.001*
CD4	854±299	443±169	<0.001*
CD8	665±386	369±151	<0.001*
CD38	609±248	318±105	<0.001*
CD16	407±266	209±129	<0.01*
CD19	175±19	117±115	0.31
CD95	1086±394	556±165	<0.01*
CD25	170±146	95±49	0.09

* p <0.05

Comparative analysis of Ly subsets between groups I and II was performed. Each group was subdivided into non-infected, latent and active HHV-6 or HHV-7 infected patient subgroups. The patients with a normal Ly count (group I) and active viral infection had a tendency for CD3+, CD4+, CD8+, CD19+ and CD95+ cell counts to increase (Table 3).

Table 3. Average count of immunocompetent cells in the GI patients group I (Ly >1400)

HHV-6/HHV-7 infection	Mo	CD3	CD4	CD8	CD19	CD16	CD38	CD95	CD25
negative	574 ±168	1489 ±660	857 ±407	616 ±408	164 ±83	430 ±287	618 ±252	1065 ±436	150 ±93
latent	576 ±170	1461 ±335	795 ±234	634 ±188	156 ±80	388 ±272	592 ±272	1013 ±259	188 ±186
active	622 ±278	1789 ±635	976 ±259	789 ±619	241 ±188	421 ±240	638 ±193	1267 ±535	152 ±82

The number of CD4+ T as well as CD19+ B cells in the patients with a lower Ly count (group II) and active HHV-6, HHV-7 infection tended to decrease (Table 4).

Table 4. Average count of immunocompetent cells in the GI patients group I (Ly < 1400)

HHV-6/HHV-7 infection	Mo	CD3	CD4	CD8	CD19	CD16	CD38	CD95	CD25
negative	493 ±244	781 ±200	446 ±199	358 ±142	120 ±52	238 ±40	337 ±136	521 ±169	101 ±58
latent	413 ±102	890 ±166	479 ±76	404 ±147	118 ±52	159 ±31	285 ±54	644 ±145	76 ±29
active	460 ±142	758 ±264	400 ±164	356 ±189	107 ±71	197 ±35	312 ±58	543 ±165	103 ±38

The GI cancer patients with latent HHV-6, HHV-7 infection had the highest number of CD25+ T cells and the lowest number of CD4+ T cells among the patients in group I; however, a significant difference of absolute count of immunocompetent cells was not observed (Table 3). In contrast with group I, the immunocompromised patients (group II) with latent beta-herpesvirus infection had the highest number of CD4+ T cells and the lowest number of CD25+ T cells between the subgroups (Table 4).

There was no considerable difference between the negative, latent and active HHV-6, HHV-7 infection subgroups in both immunocompetent and immunocompromised GI cancer patients groups. Different immune response was observed among the patients in the same subgroup.

There was no significant change in the serum level of IL-6 and s IL2R between the patients with active or latent virus infection and non-infected individuals as well. TNF-alpha level was considerable lower in the patients with latent HHV-6/HHV-7 infection in comparison with the non-infected patients (11.0±3.5 pg/ml and 15.4±5.7 pg/ml respectively).

DISCUSSION

Despite the immune response HHV-6, HHV-7 viruses are never completely eradicated from the host. Modulation of functional properties of host immune factors is an important mechanism of evading the immune response or creating an environment in which the virus can survive [15]. Our results show that patients with latent and active infection in comparison to negative had a tendency to have a higher number of the main T cell subsets and the CD95+ cells. It may be explained by additional activation of immune cells due to viral infection. In beta-herpesviruses-infected patients persistent immune activation driven by a constant supply of HHV-6 and HHV-7 antigens in chronic or latent infection was observed [16]. In comparison, our data showed that the patients with latent infection had in average higher CD3, CD4, CD8, CD38, CD95, CD25 absolute count than the patients with negative HHV-6 and HHV-7 infection. Reduced Th1 immune response was observed in HHV-6 infected individuals [17]. We did not observe Th1 immune answer depression in our infected patients. Depression of cellular immunity could be observed at various stages of cancer and the absolute count of lymphocytes in peripheral blood could be the first and simple indicator of the immune system insufficiency. Therefore, we also examined our groups of patients with different lymphocyte absolute counts. We found out that the GI patients with Ly < 1400 and latent or active infection had suppressed inductor phase of cellular immune response and a tendency to weaken humoral immune reactions. It has been observed that CD4+ T cells from HHV-6-infected individuals exert suppressive activity on the proliferation of native T cells [16]. The authors suggested that the suppressive capacity of these CD4+ T cells could be attributed to a high population of CD4+25+ T regulatory cells, which could be actively suppressing the immune response. Our data reveal that the patients with latent infection had elevated CD 25+ cell absolute count, in comparison with the negative and active infected patients. It is difficult to speculate on the role of CD 25+ in our case because to detect the exact subset of T cells with the applied method was not possible. Saff, et al. (2004) suggested that Fas (CD95+) or Fas-ligand (CD95-L+) deficient tumour-specific Th1 cells survive better in tumour-bearing mice [18]. This implies that Fas-mediated activation-induced cell death could be a limiting factor in effective T-cell-mediated immune surveillance and the immune response. Our HHV-infected patients had a tendency to increase absolute count of Fas receptor bearing (CD95+) cells. We could suggest that such activation may lead to immunocompetent cell apoptosis and immune response functional inability.

Gastrointestinal malignances are associated with a compromised immune system and viruses may be able to utilize cellular mechanisms responsible for the immune response inhibition. Our findings show that the patients who are immunocompromised (lymphopenic) and had HHV-6, HHV-7 infection had a more severe immune response deviation than the patients who are not immunocompromised. Virus-mediated immune response inhibition seems to be similar with cancer mediated. The average count of Ly subsets in this group of patients did not reflect individual immune reactions. The combinatorial study of both virus and cancer mediated immune suppressive mechanisms will help us to understand the complicated host-tumour interactions *in vivo*.

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EFFECT OF GENERAL AND REGIONAL ANAESTHESIA ON ACTIVATION OF BETA-HERPESVIRUSES, IMMUNE RESPONSE AND POSTOPERATIVE PERIOD IN PROLONGED RECONSTRUCTIVE SURGERIES

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INTRODUCTION

Beta-herpesviruses HHV-6 and HHV-7 are ubiquitous immunomodulating viruses after primary infection infecting individuals persistently throughout the life. The stimuli for reactivation of these viruses are uncharacterized, but are likely to include immunosuppression. Clinical and experimental evidence indicates that HHV-6 and HHV-7 can interfere with the function of the host immune system through a variety of mechanisms. HHV-6 and HHV-7 infect cells of the immune system as an integral part of their life cycle. By infecting immune cells of both innate and adaptive immune responses, these two viruses have the potential to impair the host defence system seriously. HHV-7 selectively infects $CD4^+$ cells, and the CD4 molecule is required for virus entry, whereas HHV-6 can infect $CD4^+$, $CD8^+$, γ/δ T cells, NK cells, *in vitro* immortalized B cells, and mononuclear phagocytes albeit unproductively. These two viruses exert differential effects on $CD4^+$ cells. Infection with HHV-6 resulted in a decrease in the T cell receptor-associated molecule CD3 whereas HHV-7 infection causes a marked decrease in the expression of CD4 molecule (Ward and Roizman, 1998).

Reconstructive free flap surgery is a complex method of wound closure for large wounds not amenable to linear (primary) closure. It involves the transfer of free tissue (muscle, bone or a combination) to a site of the tissue loss where its circulation is restored via microvascular anastomoses. Usually patients requiring reconstructive flap surgery are in the working age without significant co-morbidities. Regardless of the young age and adequate previous medical condition of patients, considerable differences of clinical course after the trauma and the postoperative period after free flap surgery such as surgical outcome, complication rate and duration have been observed. In our study patients anaesthesia and surgery are long lasting (5–10 h).

Anaesthesia proves to be an essential factor suppressing the immune system, in particular cell-mediated immunity, in the post-operative period. Anaesthesia-associated immunomodulation and intensification of immunosuppressive effect by beta-herpesviruses activation can increase the susceptibility of patients to other infections such as bacterial, fungal infections and provoke postoperative complications, e. g., wound-healing disturbances and infections, leading to sepsis, followed by multiple organ failure and potential lethal outcome (Homburger and Meiler, 2006; Mamaja, et al., 2008).

The aforementioned differences during a postoperative period course have stimulated research on how the prolonged reconstructive flap surgery and anaesthesia technique might impact reactivation of beta-herpesviruses and how activation of these viral infections is associated with the nearest postoperative period course.

AIM

This study aimed to investigate the presence of human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) in patients before a prolonged reconstructive flap surgery and the effect of prolonged reconstructive flap surgery upon general and regional anaesthesia on activation of human herpesvirus 6 (HHV-6) and human herpesvirus 7 (HHV-7) infections and how this activation is associated with a postoperative period course.

MATERIALS AND METHODS

A retrospective analysis was done for 38 patients (aged 5–65) who underwent long lasting (average 5.7h) reconstructive flap surgery procedures in The Centre of Plastic and Reconstructive Microsurgery of Latvia. The cohort was established with the approval of the Ethics Committee of Rīga Stradiņš University and all participants gave their informed consent prior to the examination. All our study patients in between the trauma and the reconstructive surgery had had previous surgeries (GA average 2.75 and RA average 1.28). Causes of the primary defect were extensive surgery (breast cancer), upper extremity trauma, lower extremity trauma, congenital defects.

The patients were split into two study groups according to the anaesthesia method applied – general anaesthesia (GA) group, and regional anaesthesia (RA) group. For 17 patients GA and for 21 patients RA was used. The patients were not randomized depending on the anaesthesia method but surgical necessity, priority was given to regional anaesthesia.

EDTA anti-coagulated peripheral blood samples were collected from the patients before and 10 days after the anaesthesia and reconstructive flap surgery. Total DNA was extracted from peripheral blood leukocytes (PBL) and cell-free blood plasma. Nested polymerase chain reaction (nPCR) was used for the detection of HHV-6 and HHV-7 sequences in PBL and plasma DNAs. The detection of HHV-6 DNA was carried out as described before (Chapenko, et al., 2004; Mamaja, et al., 2007). The presence of viral sequences in PBL DNAs was a marker of latent/persistent viral infection and in plasma DNAs – of active viral infection (plasma viremia). Peripheral blood CD4+, CD8+, CD38+, and CD16+ positive cells were detected by laser flow cytofluorometer (FACS, Calibur, USA, Becton Dickinson) using corresponding monoclonal antibodies.

Duration of the postoperative period, the time spent in the intensive care units (ICU), and the number of repeated and unfavourable surgeries after the reconstructive flap surgery, the therapy outcome in relation to the anaesthesia method and activation of β-herpesviruses were assessed.

RESULTS

Before the surgery latent/persistent HHV-6 infection was revealed in 14 out of 38 patients (36.84%) (8 patients in the GA group, and 6 – in the RA group) and active HHV-6 infection – in 3 out of 38 patients (7.89%) (2 patients in the GA group, one – in the RA group). In 21 out of 38 patients (55.27%) HHV-6 infection was not detected. At the same time latent/persistent HHV-7 infection was revealed in 23 out of 38 patients (60%) (10 patients in the GA group, and 12 – in the RA group) and active HHV-7 – in 8 out of 38 patients (21%) (4 patients in the GA group, 4 – in the RA group). In 6 out of 38 patients (15.79%) HHV-7 infection was not detected. In 8 patients (5 patients in the GA group, and 3 patients – in the RA group) concurrent latent/persistent HHV-6/HHV-7 infection was found and in one of them (in the GA group) concurrent infection was active (Figure1, Table 1).

After the surgery latent/persistent HHV-6 infection was detected in 13 out of 38 patients (34.31%) (7 patients in the GA group, and 6 – in the RA group) but active HHV-6 infection – in 4 out of 38 patients (10.51%) (3 patients in the GA group, one – in the RA group). 21 out of 38 patients (55.27%) was HHV-6 infection free. Whereas latent/persistent HHV-7 infection was revealed in 19 out of 38 patients (50%) (7 patients in the GA group, and 12 – in the RA group) and active HHV-7 infection – in 12 out of 38 patients (31.57%) (8 patients in the GA group, and 4 – in the RA group), but 6 out of 38 patients (15.79%) were HHV-7 infection free. In 8 patients (4 patients in the GA group, and 4 – in the RA group)

concurrent latent/persistent HHV-6/HHV-7 infection was detected, but in one patient in the GA group concurrent infection was active (Figure 1, Table 1).

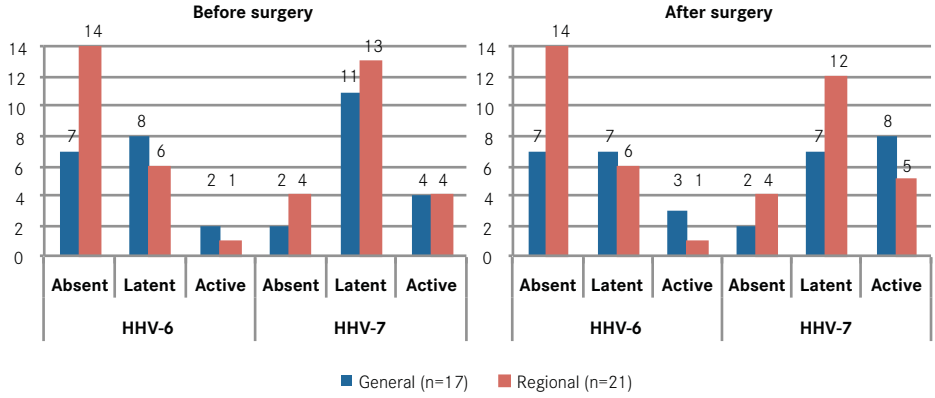

Figure 1. Frequency of latent/persistent and active HHV-6 and HHV-7 infection before and after anaesthesia and surgery

Table 1. Percentage of latent/persistent and active HHV-6 and HHV-7 infection before and after anaesthesia and surgery

	Before the surgery		After the surgery	
	Latent/persistent	Active	Latent/persistent	Active
HHV-6	36.84%	7.89%	34.31%	10.51%
HHV-7	60%	21%	50%	31.57

After long lasting reconstructive free flap surgery in one patient of the GA group reactivation of HHV-6 infection was detected, contrary to the RA group where no cases of reactivation were revealed. At the same time in 5 patients (4 patients in the GA group, and one – in the RA group) reactivation of HHV-7 infection was detected. In one patient in the GA group simultaneous activation of HHV-6 and HHV-7 was detected after the surgery whereas in the RA group simultaneous activation of HHV-6 and HHV-7 was not revealed.

In the RA group in comparison with the GA group the quantity of activated lymphocytes was initially significantly lower, but in the postoperative period the immune response showed statistically significant increase of activated lymphocytes CD38+ ($p<0,05$). Also the tendency of decrease of T helper subset (CD4+) was observed (Table 2, 3).

Table 2. Assessment of the immune response for all RA group before and after surgery

	Lymphocytes	CD4	CD8	CD38	CD16	CD4/CD8
Before	1668.85±606,08	837.32±486,1	435.89±203	508.95±184.6	221.53±121.46	1.97±0.14
After	1622.63±450	773.79±217.8	388.74±130.7	626.16±196*	267.42±187.95	2.10±0.16

Data are means and SD;* $p<0.05$

Table 3. Assessment of the immune response for all GA group before and after surgery

	Lymphocytes	CD4	CD8	CD38	CD16	CD4/CD8
Before	1733.82±618.42	801.53±381.6	467.29±203	529.94±257.5	223.41±143.1	1.86±0.1
After	1689.12±548.3	780.31±241.3	429.44±130	665.94±227.9	266.53±171.3	2±0.12

In the GA group 6 cases of unfavourable surgery results were observed: 4 cases – surgical site infection, 2 cases – flap ischemia. In patients of the RA group 2 cases of unfavourable surgery results were observed – both surgical site infection. A significant relationship between active HHV-6 viral infection before reconstructive flap surgery and postoperative surgical site infection was observed irrespective of the anaesthesia technique applied.

Both patients of the RA group before the reconstructive flap surgery were suffering from osteomielitis and also the postoperative period proceeded with complication. In one patient from the GA group with detected reactivation the postoperative surgical site infection was observed as well (Table 4).

Table 4. The main characteristics of postoperative period

	General Anaesthesia	Regional Anaesthesia
All patients	**(n=17)**	**(n=21)**
Number of unfavourable surgeries	6	2
• Surgical site infections	4	2
• Flap ischemia	2	0
Number of repeated surgeries until the second blood sample (n)	2.38±0.9	1.2±0.3
Time spent in ICU (days)	4.17±9.77	2
Duration of postoperative period (days)	23.76±20.75	11.5±8.76

Data are means and SD

DISCUSSION

HHV-6 and HHV-7 can be reactivated in immunosuppressed states. HHV-6 itself affects almost all components of the immune system, including both innate and adaptive immune functions. HHV-6 replicates in and kills CD4+ and CD8+ T-cells. HHV-6 viral envelope proteins inhibit T lymphocyte proliferation induced by phytohemagglutinin (PHA), IL-2, or antigens (Horvat, et al., 1993). Interaction of inactivated HHV-6 viral particles with PBMC inhibits proliferation of both CD4+ and CD8+ T lymphocytes and their responses to IL-2. This defect is apparently due to the induction of defective IL-2 receptors or defects in IL-2 induced signalling pathway in these cells, as exogenous IL-2 does not correct the HHV-6 induced proliferation defect (Flamand, et al., 1995). In our study we observed that patients with active HHV-6 infection before reconstructive flap surgery had infectious complications, which could be related to immunomodulatory properties of HHV-6. Numerous studies (Kurosawa and Kato, 2008) have shown that alongside with immune suppression caused by surgical stress, anaesthetics and analgesic agents commonly used in surgery and in intensive care may directly affect the functions of immune-competent cells. Since the reactivation of HHV-6 and HHV-7 viral infection is more pronounced in patients of the GA group, our data are consistent with the previous studies that GA does not suppress the surgical stress response, thus exacerbating postoperative immunosupression (Stevenson, et al., 1990). Immunosupression is the main cause of the reactivation of HHV-6 and HHV-7 viral infection. Spinal anaesthesia results in less immunosupression, i. e. it maintains the number of Th1 cells, thus stimulating cell immunity. The effects are most pronounced in high risk

patients undergoing procedures below the umbilicus (Liu, et al., 1995). Serious disorders of the immunological system may cause complications, as there are disorders in wound healing, increased number of infections, inadequate response to stress, multiorganic suppression and increased incidence of metastases (Rosen, et al., 1992). The data of our study show that reconstructive flap surgery under GA could significantly increase beta-herpesviruses activation frequency in comparison with long lasting reconstructive surgery with RA.

GA could lead to suppression of cellular immune response in patients with latent viral infection and significantly increase the frequency of virus reactivation in comparison with RA. This may cause more difficult postoperative period and patients' recovery.

CONCLUSION

To the best of our knowledge, this is the first study documenting the presence and activation of HHV-6 and HHV-7 infection in patients undergoing prolonged reconstructive surgery. Despite the limited number of patients our study results suggest that the presence of HHV-6 and HHV-7 infection in our study group was significantly high.

Reactivation of HHV-6 and HHV-7 infection is more frequent in patients to whom general anaesthesia is applied. Our results suggest that reactivation of HHV-6 and HHV-7 infection is possibly related to longer and more complicated postoperative period with a worse clinical outcome.

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FREQUENCY OF HHV-6 AND HHV-7 INFECTION MARKERS IN RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS PATIENTS

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INTRODUCTION

Human herpesvirus (HHV)-6 and HHV-7 are closely related viruses classified into the *Roseolovirus* genus in the Betaherpesvirinae subfamily. HHV-6 and HHV-7 are ubiquitous in general population, with up to 90% of adults being seropositive. They both are the causative agents of exanthema subitum. Like other herpesviruses, after primary infection, which commonly occurs in early childhood, they undergo latency. Both viruses may become reactivated under conditions of stress and immunocompromised state, and this reactivation may lead up to immunosupression (Caselli & Luca, 2007). Both the viruses infect cells of the immune system and therefore can modulate their function. The primary target for HHV-6 and HHV-7 replication is CD4+ T lymphocytes, pivotal cells in generation of humoral and cell-mediated adaptive immune response. Both viruses have shown to increase TNF-alpha secretion by PBMC. HHV-7 could also act as a trigger factor for HHV-6 activation (Lusso, 2006; Wang & Pellet, 2007, Prober, 2011).

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown aetiology, characterized by chronic inflammation of synovium and subsequent joint destruction. It has been suspected that viral infections could be involved in the aetiology and pathogenesis of RA either *via* direct joint tropism that cause tissue damage or *via* their ability to activate the immune response directed at joint tissue. Infiltration of various types of leukocytes is involved in joint inflammation, and leukocyte accumulation has been found out to play a critical role in the pathogenesis of RA. There are several reports supporting the presence of certain viruses including HHV-6 as potential triggers of RA on the basis of epidemiological evidence, however, the role of HHV-6 in RA remains unclear. To assess the relevance of viral infection in RA we evaluated the frequency of HHV-6 and HHV-7 reactivation in RA patients and compared it with that in osteoarthritis (OA) patients and apparently healthy persons.

MATERIALS AND METHODS

Patients and controls. HHV-6 and HHV-7 infections have been studied in 35 patients with RA (27 females and 8 males) with mean age 56.1 years (range 38–76), 33 patients with OA, 25 females and 8 males with mean age 66.1 years (range 46–83) as well as 31 apparently healthy persons (mean age 53.6 years, range 38–72).

The clinical features and laboratory parameters (hemoglobin, C reactive protein, rheumatoid factor and anti-cyclic citrullinated peptide) and disease activity score *DAS28* (only for RA patients) were detected and analysed to ascertain their potential relationship with HHV-6 and HHV-7 infections.

Sample collection and preparation. EDTA anti-coagulated peripheral blood, synovial tissues and synovial fluid samples from RA and OA patients and EDTA anti-coagulated peripheral blood samples from apparently healthy persons were collected. Plasma samples were separated from peripheral blood by centrifugation.

DNA was isolated from peripheral blood leucocytes (PBL), cell-free plasma, synovial tissues and synovial fluid by standard phenol-chloroform extraction. Before DNA extraction plasma and synovial fluid samples were treated with

DNase I. To assure the quality of the PBL and synovial tissue DNA as well as to exclude contamination of plasma and synovial fluid DNA by cellular DNA a beta-globin PCR was carried out.

HHV-6 and HHV-7 detection by nested PCR

One microgram of PBL and synovial tissue DNA samples as well as 10 μl of plasma and synovial fluid DNA was subjected to nested PCR with HHV-6 and HHV-7 specific primers as described previously (Secchiero, et al., 1995; Berneman, et al., 1992). HHV-6 and HHV-7 positive and negative DNAs as well as water controls were included in each experiment. Detection of viral DNA in cell-associated material – leucocytes and synovial tissues – has been used as a marker of latent/persistent infection while detection of viral DNA in cell-free plasma and synovial fluid as a marker of active infection (virus reactivation).

Statistical analysis

The data were analyzed by Fisher's exact test. Values of $p<0.05$ were considered to be significant.

RESULTS

Prevalence of latent/persistent HHV-6 and HHV-7 infections. Latent/persistent HHV-6 or/and HHV-7 infection was observed in 33/35 (94.3%) RA and 31/33 (93.9%) OA patients as well as in 25/31 (80.6%) healthy persons. 13/35 (37.1%) RA patients, 20/33 (60.6%) OA patients and 11/31 (35.4%) healthy persons presented HHV-6 infection. At the same time HHV-7 infection was revealed in 30/35 (85.7%) patients with RA, 30/33 (90.9%) – with OA and in 21/31 (67.7%) apparently healthy persons. Among RA patients 3/35 (8.6%) had single HHV-6 infection, 20/35 (57.1%) – single HHV-7 infection and 10/35 (28.6%) – dual HHV-6 and HHV-7 infection. Among OA patients single HHV-6 infection was detected in one of 33 patients (3.0%), single HHV-7 infection – in 11/33 (31.4%) patients and dual HHV-6 and HHV-7 infection – in 19/33 (57.6%) patients. Incidence of HHV-6, HHV-7 and concurrent HHV-6 and HHV-7 latent/persistent infection among apparently healthy persons was 3/31 (9.7%), 13/31(41.9%) and 8/31 (25.8%), respectively. In two RA (5.7%) and two OA patients (6.1%) as well as 7 (22.6%) apparently healthy persons HHV-6 and HHV-7 infection was not revieled.

Frequency of HHV-6 and HHV-7 reactivation

On the whole, HHV-6 reactivation had been detected in 9/13 patients with RA (69.2%) and 17/20 (85.0%) with OA patients, as well as in 3/11 (27.3%) apparently healthy persons. Single HHV-6 reactivation was observed in 3/13 (23.1%) of RA patients with HHV-6 latent/persistent infection [2/3 (66.6%) with single HHV-6, and 1/10 (10.0%) with dual HHV-6 and HHV-7 infection], in 2/20 (10.0%) of OA patients [1/1 with single HHV-6 infection and 1/19 (5.3%) with dual HHV-6 and HHV-7 infection] and in none of healthy persons. HHV-7 reactivation was observed in 24/30 (80.0%) of RA patients, 27/30 (90.0%) of OA patients and in 4/21 (19.0%) apparently healthy persons. Single HHV-7 reactivation had been detected in 17/30 (56.6%) of RA patients [16/20 (80.0%) with single HHV-7 infection and 1/10 (10.0%) with dual HHV-6 and HHV-7 infection], 10/30 (33.3%) of OA patients (9/11 with single HHV-7 infection and 1/19 with dual HHV-6 and HHV-7 infection) and 1/21 (19.0%) of apparently healthy persons with single HHV-7 infection. Concurrent HHV-6 and HHV-7 reactivation had been observed in 7/10 (70.0%) of RA patients, 16/19 (84.2%) of OA patients and 3/8 (37.5%) of apparently healthy persons with dual HHV-6 and HHV-7 latent/persistent infection. In six RA patients (one with single HHV-6, 4 – with single HHV-7 and one with concurrent HHV6 and HHV-7), in 3 OA patients (two with single HHV-6 and one – with concurrent HHV-6 and HHV-7) as well as in 20 apparently healthy persons with latent/persistent herpesviruses infection the HHV-6 or HHV-7 reactivation was not found.

Presence of HHV-6 and HHV-7 DNA in synovial fluid

HHV-6 and/or HHV-7 genomic sequences were found in synovial fluid of 5/6 (83.3%) RA and 11/12 (91.7%) OA patients. Single HHV-6 was detected in 2/6 (33.3%) samples of the RA patients and 1/12 (8.3%) of the OA patients while single HHV-7 genomic sequence had been found in 2/6 (33.3%) samples of the RA and 5/12 (41.7%) of OA patients (Table 2). Concurrent presence of HHV-6 and HHV-7 genomic sequences in synovial fluid samples had been observed in 1/6 (16.7%) of the RA and 5/12 (41.6%) of OA patients (Table 1).

Presence of HHV-6 in synovial tissues

Presence of HHV-6 genomic sequence had been detected in 3/7 synovial tissues samples of the RA (42.8%) and in 12/24 (50.0%) of OA patients (Table 1).

Table 1. Frequency of herpesvirus HHV-6 and/or HHV-7 DNA in synovial tissues and synovial fluid of the RA and OA patients

Patients	Synovial fluid			Synovial tissues
	HHV-6	HHV-7	HHV-6/ HHV-7	HHV-6
RA patients	2/6 (33.3%)	2/6 (33.3%)	1/6 (16.7%)	3/7 (42.9%)
OA patients	1/12 (8.33%)	5/12 (41.7%)	5/12 (41.7%)	12/24 (50.0%)

Disease activity and clinical parameters of the RA and OA patients with and without HHV-6 and HHV-7 infection. The results of clinical investigation of the RA and OA patients are shown in Table 2 and 3, respectively. Analysis of clinical parameters showed that the disease activity and aggressiveness in the RA patients with latent/ persistent HHV-6 and/or HHV7 infection were significantly higher. The RA patients with concurrent HHV-6 and HHV-7 reactivation have presented more active and more aggressive disease course in comparison with the RA patients with single HHV-6 or HHV-7 reactivation. In the RA patients with active HHV-7 infection a more common complication was anemia. However, there was no significant correlation between the herpesviruses reactivation and the disease activity in the RA as well as in OA patients.

Table 2. Medium results of laboratory parameters and disease activity in RA patients.

Infection	C reactive protein (mg/L)	DAS28	RF (U/ml)	Anti CCP (RU/ml)	Hb (g/L)
Without infection	35.7	4.72	117.4	1.8	123
Latent persistent HHV-6 and/or HHV7	20.3	5.61	347.27	136.27	127.5
Active HHV-6	26.9	4.64	171.7	49.7	125.3
Active HHV-7	11.88	4.51	142.56	83.2	119.16
Active HHV-6/HHV-7	6.56	4.77	249.5	217.9	128.43

Table 3. Medium results of laboratory parameters in OA patients.

Infection	C reactive protein (mg/L)	RF (U/ml)	Anti CCP (RU/ml)	Hb (g/L)
Without infection	3.88	6.64	7.64	140.35
Latent persistent HHV-6 and/or HHV7	2.4	7.7	5.17	133
Active HHV-6	4.45	3.5	7	151
Active HHV-7	4.8	8.73	8.2	138.75
Active HHV-6/HHV-7	2.89	9.63	7.72	138.67

DISCUSSION

In this study we analyzed the frequency of HHV-6 and HHV-7 reactivation among the RA patients by nested PCR in order to find out the possible relationship between the infection and the disease. As the control groups the OA patients and apparently healthy persons were used. The results showed that the prevalence of latent/persistent HHV-6 and HHV-7 infections among the RA and OA patients and apparently healthy persons were similar. The frequency of HHV-6 and HHV-7 reactivation (active infection) was significantly higher in the RA and OA patients compared to apparently healthy persons ($p=0.0377$ and $p=0.00073$ for HHV-6 and $p=0.000349$ and $p=0.0000102$ for HHV-7, respectively). These observations are in accordance with the findings of other authors (Alvarez-Lafuente, et al., 2005; Newkirk, et al., 1994). However, no differences in the frequency of HHV-6 and HHV-7 reactivation are observed between the RA and OA patients. The single HHV-6 reactivation was found in the RA and OA patients only and HHV-6 reactivation frequency was similar. The single HHV-7 reactivation has been observed in the RA and OA patients as well as in apparently healthy persons. The frequency of HHV-7 reactivation was significantly higher in the RA and OA patients compared to apparently healthy persons ($p=0.0001$ and 0.0056, respectively). In the RA patients single HHV-7 reactivation was detected more frequently than in the OA patients, however, this difference was not significant. The frequency of concurrent HHV-6 and HHV-7 reactivation was significantly higher in the OA patients compared to apparently healthy persons ($p=0.0022$). There were no significant differences in the frequency of concurrent HHV-6 and HHV-7 reactivation between the RA and OA patients. The prevalence of HHV-6 DNA in synovial tissues of the RA and OA patients was also similar. There were no significant differences between the RA and OA patients in the frequency of single HHV-6 and HHV-7 as well as concurrent HHV-6 and HHV-7 reactivation in synovial fluid. No significant correlation of herpesviruses reactivation with disease activity has been found.

Thus, although the frequency of HHV-6 and HHV-7 reactivation was significantly higher in the RA patients compared to apparently healthy persons, there were no differences in the frequency of HHV-6 and HHV-7 reactivation between the RA and OA patients and association of the herpesviruses reactivation with the disease activity was not found. These findings suggest that the viral reactivation is a consequence, rather than the cause of RA. It is more likely that RA and OA patients may be more prone to reactivation of these viruses as a consequence of drug treatment or defect in cellular immunity. The majority of the RA patients with HHV-6 or/and HHV-7 reactivation received methotrexate. Earlier the reactivation of latent Epstein-Barr virus by methotrexate in RA patients has been described by Feng, et al., 2004. Nevertheless, we could not exclude that these viruses have a potential role in the development of RA through molecular mimicry or disregulation of leukocyte functions. Both HHV-6 and HHV-7 may cause functional disturbance of the immune system and induce differential secretion of a wide array of cytokines and chemokines, including tumour necrosis factor alpha which play a crucial role in RA. In addition, HHV-6 can activate other herpesviruses such as EBV (Flamand, et al, 1993).

CONCLUSION

There was no correlation of HHV-6 and/or HHV-7 reactivation with the disease activity of RA, but a potential role of these viruses in pathogenesis of RA through molecular mimicry, immunedisregulation and activation of other viruses could not be excluded.

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ROLE OF T-CELL SUBSETS IN REGULATION OF IMMUNE RESPONSE

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T cells are a subset of lymphocytes that play an important role in the immune response and is at the core of adaptive immunity. The main function of T-cells is to fight virus-infected and cancer cells. The basic types of mature T cells are T helper CD4+ and T cytotoxic lymphocytes (CTL) CD8+.

CD4+ T helper (T_H) lymphocytes are essential regulators of immune response and play a central role in immune protection. They do so *via* their capacity to help B cells make antibodies, to induce macrophages to develop enhanced microbicidal activity, to recruit neutrophils, eosinophils, and basophils to the sites of infection and inflammation, and, through their production of cytokines and chemokines, to orchestrate the whole immune system. These cells are also known as $CD4^+$ T cells because they express the CD4 protein on their surface. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of Antigen Presenting Cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response.

T_H cells differentiate into effector cells specialized in cytokine secretion and function. Today at least 4 distinct CD4+ T-cell subsets have been proved (?) described existent, T_H1, T_H2, T_H17, and Treg cells. T_H1 cells produce interferon-γ (IFN-γ) and mediate cellular immunity, whereas T_H2 cells produce interleukin 4 (IL-4), IL-5 and IL-13 and mediate humoral immunity and allergic responses. Besides T_H1 and T_H2 cells, T_H17 cells constitute a third subset of T helper cells with distinct effector functions. The differentiation factors (TGF-β plus IL-6 or IL-21) and specific transcription factors (STAT3, IRF4, RORγt, and RORα) that define the T_H17 transcriptional programme have been identified. T_H17 cells secrete not only IL-17 but also IL-17F, IL-21, and IL-22, these cytokines most likely cooperate to induce tissue inflammation, and T_H17-driven effector functions may be different in different tissues. T_H17 T cells have become notorious for their involvement in a range of autoimmune diseases, but an exclusive role as mediators of pathology is unlikely to be their primary function. IL-17 stimulates the mobilization and *de novo* generation of neutrophils by granulocyte-colony stimulating factor (G-CSF), thereby bridging innate and adaptive immunity. It has been suggested that this might constitute an early defense

mechanism against severe trauma that would result in tissue necrosis or sepsis. The primary function of T_H17 cells appears to be the clearance of extracellular pathogens during infections. However, T_H17 cells also promote inflammation and have been implicated in the pathogenesis of many experimental autoimmune diseases, as Crohn's disease (an inflammation of the small intestine), ulcerative colitis (inflammation of the large intestine), psoriasis (inflammation of the skin), an animal model (in mice) of multiple sclerosis and rheumatoid arthritis.

Regulatory T cells (Tregs) have the ability to suppress the activity of most other lymphoid cells, as well as dendritic cells through cell–cell contact-dependent mechanisms, which have not yet been fully defined. Tregs are a key component of a functional immune system and Treg deficiency is associated with severe autoimmunity and allergies. However, Tregs specific for tumour-associated antigens are present in cancer patients and Tregs accumulate in many types of solid tumours, where they probably act to promote tumour escape from cytotoxic immune responses. Naturally occurring $CD4^+CD25^+Foxp3^+$ Treg (nTreg) derive from the thymus and constitute 3–10% of the naïve peripheral $CD4^+$ T cell population in humans. As CD25 is also upregulated on the surface of activated effector T cells, other specific markers are needed to identify nTreg. To date, the best marker of nTreg is the intracellular expression of the transcription factor forkhead box P3 (Foxp3).

CD8+ cytotoxic T lymphocytes (CTL) are principally known for their role in cytotoxic killing of virus-infected and tumour cells and play an important role of cellular immune system. They receive activation signal when their T-cell receptor (TCR) and the CD8 coreceptor recognizes MHC-I-bound peptide antigen, presented on all nucleated cells surface. But CTL cells recognize tumours cells, when their T-cell receptor (TCR) recognizes MHC-I-bound peptide antigen, presented on the surface of professional antigen-presenting cells (pAPCs), namely dendritic cells (DCs) and macrophages/monocytes. Tumours cells lack co-stimulatory signal, which is generally indispensable for their full activation and survival. The delivery of appropriate activation signals to naïve CD8+ T cells leads to their proliferation and concomitant differentiation into cytotoxic T lymphocytes (CTL), which die by apoptosis after elaborating their effector functions, and memory CD8+ T cells (both central and effector), which are generated in much smaller quantities and are retained for fighting potential subsequent exposure to the same antigens, eliciting a more rapid and aggressive response. Activated CD8+ T cells are able to induce cytolysis of infected cells by two distinct molecular pathways: the granule exocytosis pathway, dependent on the pore-forming molecule perforin, or by the upregulation of FasL (CD95L), which can initiate programmed cell death by aggregation of Fas (CD95) on target cells. Both of these pathways, activated in response to signals from the TCR, stimulate the caspase cascade in the target cell, leading to apoptotic death. Efficient lysis by the granule exocytosis pathway requires the coordinated delivery of perforin and granule enzymes, such as granzymes A and B, into the target cell. CD8+ effector cells fall into two subpopulations based on cytokine secretion. Type 1 CD8+ T cells (Tc1) secrete IFN-γ, whereas type 2 CD8+ T cells secrete IL-4, IL-5 and IL-10. CD8+ T cells also elaborate cytokines, including IFN-γ and TNF, as well as chemokines that function to recruit and/or activate the microbicidal activities of effector cells such as macrophages and neutrophils. Cytokines may also directly interfere with pathogen attachment or pathogen gene expression, or they may restrict intracellular replication. As with cytolytic effector mechanisms, expression of cytokine molecules by CD8+ T cells is tightly regulated through TCR-dependent signals.

CD44 is a marker of a family of 85–200kDa transmembrane glycoproteins that are widely expressed in a variety of cell types. Effectors and memory cells express high levels of CD44 antigen. $CD44^h$ is recognized as a marker of memory phenotype and also as an activation marker of naïve T lymphocytes. CD44+ T lymphocytes may also be involved in tumour growth. The aim of the study was to evaluate the role of $CD8^+CD44^h$ lymphocytes during growth of SL2 tumours in DBA/2 mice. Two SL2 tumours were implanted by subcutaneous injection of 10^7 tumour cells in the same DBA/2 mouse with the interval of 2 days. We evaluated the numbers of $CD8^+CD44^h$ cells in peripheral blood of DBA/2 mice by flow cytometry (Figure1B) and compared with the numbers of these cells in the control mice. We also investigated the influence of $CD8^+CD44^h$ cells on the growth of SL2 tumours. The results of our study show (Figure 2) that the percentages of $CD8^+CD44^h$ cells in the CD8+ subset were significantly increased in peripheral blood of DBA/2 mice compared with the control mice (59.33% ± 20.54% versus. 20.97% ± 5.14%, $p = 0.0093$).

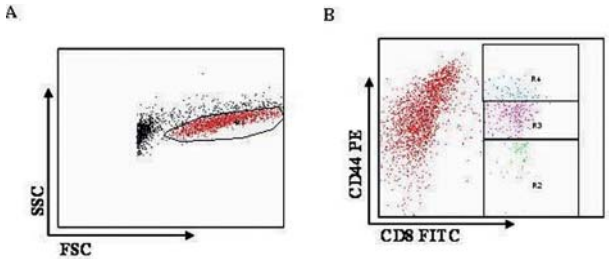

Figure 1. **CD8$^+$CD44^h cells in the peripheral blood in DBA/2 mice**.(A) by SSC/FSC dot plot lymphocyte (R1) were separated and CD44 expression on CD8$^+$ T lymphocytes was analyzed in the CD8/CD44 dot plot (B). Cell population with a high expression of CD44 marker (R4) in the CD8+ T cells was identified.

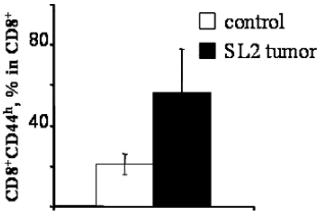

Figure 2. **Percentage of CD8$^+$CD44^h cells of the total numbers of CD8$^+$ T cells in the peripheral blood of control (empty bar) and SL2 tumours mice (black bar) on day 9 after SL2 tumours implantation**. Percentage of CD8$^+$CD44^h cells in CD8+ subset was significantly increased in SL2 tumour-bearing mice (**p = 0.0093). Values are given as means ± SD.

Time-course experiments also showed that the percentage of CD8$^+$CD44^h cells in the CD8+ subset in the peripheral blood increases during tumour growth (17.72% ± 5.14% on day 0, 12.13% ± 5.27% on day 5 and 27.65% ± 6.97% on day 9; the difference between day 0 and day 9 is statistically significant, (p=0.013) (Figure 3).

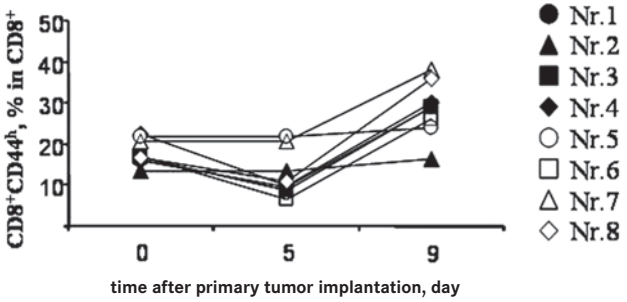

Figure 3. **Percentage of CD8$^+$CD44^h cells of the total numbers of CD8$^+$ T cells in the peripheral blood of SL2 tumours mice during tumours growth in individual mouse (n=8) on the days 0, 5 and 9 after primary tumour implantation.** CD8$^+$CD44^h cells increased on day 9 after the tumour implantation (*p=0.013).

However, no correlation was found between the changes in $CD8^{+}CD44^{h}$ cells and the weight or the volume of SL2 tumours. Thus, despite cell-mediated immunity is generated during the growth of SL2 tumours in DBA/2 mice, it does not exert a critical role upon the progression of SL2 tumours.

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OCCURRENCE OF HUMAN HERPESVIRUS 6 AND 7 INFECTION IN BELARUS

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INTRODUCTION

Human herpesvirus-6 (HHV-6) and -7 (HHV-7) belong to the family *Herpesviridae*, the subfamily *Betaherpesvirinae*, the genus *Roseolovirus* (1). The increased interest in these viruses both in the theoretical and in practical medicine is caused by the following factors: the viruses are widespread and after primary infection remain in the form of latent/persistent infection for life, and under specific conditions they cause chronic inflammatory processes; under the effect of different factors (somatical and infectious diseases, stress, immunosuppression and other) they can be activated and change the course of the basic disease (2–4). Moreover, activation of the viruses can be simultaneous and correlated with the active phase of the disease (5, 6). In recent studies viral infections are associated with pathogenesis of chronic fatigue syndrome (CFS). Some of the authors assume that the HHV-6 and HHV-7 infection are the leading factor of this disease development (7–9).

The aim of this work is detecting the occurrence frequency of HHV-6, HHV-7, their activation, correlation with different forms of the infectious process and the establishment of their possible role in the development of the of chronic fatigue syndrome.

MATERIALS AND METHODS

Patients. There were examined 105 patients with different diagnoses of the basic disease: fever of unknown etiology; acute encephalitis, multiple sclerosis, herpesviruses infection of different etiology, chronic herpesviruses infection, AIDS, hepatitis A, chronic hepatitis, chronic and acute respiratory disease.

Material. Peripheral blood and liquor taken from the patients on admission to hospital.

Molecular methods. Nested polymerase chain reaction (nPCR) was used for determining HHV-6, HHV-7 genomic sequences in peripheral blood leukocytes (PBL) and cell free plasma DNA (markers of latent/persistent and active infection, respectively). DNA was isolated from the PBL and blood serum by using the test-systems "Amplisens" (Russia) and QIAGEN (Germany).

RESULTS AND DISCUSSION

The molecular-biological monitoring of the latent/persistent and active HHV-6 and HHV-7 infection was carried out in the groups of patients who are potential carriers of these viruses infection (Table 1). Prevalence of HHV-6, HHV-7 infection was examined in patients with different pathology of infectious and neurologic profile. It revealed that more than 50% of those patients had markers HHV-6 and more than 33% – HHV-7. The conducted examination on the viral DNA detection in the PBL, plasma and liquor from the patients with different diagnoses of the basic disease revealed the presence of the β-herpesviruses infection in all groups of patients (Table 1).

Table 1. Frequency of β-herpesviruses detection in the groups of patients with different diagnoses of the basic disease

Basic disease	Patients (n)	DNA HHV-6			DNA HHV-7		
		leukocytes	plasma	liquor	plasma	plasma	liquor
multiple sclerosis	30	12/25 (48%)	9/30 (30%)	4/7 (57%)	5/25 (25%)	3/30 (10%)	3/7 (43%)
acute encephalitis	5	1/3 (33%)	1/5 (20%)	1/3 (33%)	0/3 (0%)	0/5 (0%)	0/3 (0%)
fever of unknown etiology	9	6/9 (66%)	5/9 (55%)	ни	3/9 (33%)	2/9 (22%)	ни
AIDS	2	2/2 (100%)	½ (50%)	1/1 (100%)	1/2 (50%)	0/2 (0%)	½ (50%)
chronic hepatitis	7	3/5 (60%)	1/7 (14%)	ни	1/5 (20%)	0/7 (0%)	ни
chronic acute respiratory disease	7	3/7 (43%)	1/7 (14%)	ни	1/7 (14%)	0/7 (0%)	ни
hepatitis A	24	ни	4/24 (17%)	ни	ни	1/24 (4,%)	ни
chronic herpesvirus infection	21	12/21 (51%)	10/21 (47%)	ни	5/21 (24%)	3/21 (14%)	ни
total/positive	105	37/72 (51%)	24/93 (26%)	6/11 (54%)	16/72 (22%)	9/82 (10%)	4/12 (33%)

The presence of HHV-6 genomic sequences in PBL was revealed in 12 out of 25 patients with multiple sclerosis, in 1/3 with sharp encephalitis, in 6/9 with fever of unknown etiology, in 3/5 with chronic hepatitis, in 3/7 with chronic acute respiratory infections and in 12/21 with chronic herpesvirus infections. In 2 patients with AIDS HHV-6 DNA was detected in PBL and in one of them – in the liquor. The liquor from other patients was tested. It was also revealed in 4/7 of patients with multiple sclerosis and in 1/3 with sharp encephalitis. The presence of HHV-6 DNA in PBL DNA and the liquor DNA testifies about a latent/persistent form of the infection. The active HHV-6 infection was discovered in 30% of the patients with multiple sclerosis, in one patient with sharp encephalitis and in one patient with AIDS. As a rule, the activation of the infection was observed on the background of the basic disease in the patients with neurologic symptoms in an active phase.

The presence of HHV-7 genomic sequences in PBL DNA was detected in 5/25 of the patients with multiple sclerosis and in 3 of them – also discovered in the liquor DNA (Table 1). In the patients with fever of unknown etiology HHV-7 DNA in PBL DNA was detected in 33% cases, with chronic hepatitis – in 20%, with chronic acute respiratory virus infection – in 14%, with herpesvirus chronic infection – in 24%. HHV-7 DNA was detected in PBL and in liquor DNAs of the HIV infected patient. The studies of HHV-7 plasma viremia showed that it was recorded on the background of herpesvirus chronic infection, multiple sclerosis and fever of unknown etiology in 14% of cases, in 10% and in 22% cases, respectively.

There was also performed examination of the markers of herpesviruses 4 (EBV) and 5 (CMV) infection for the definition of mix infections in the patients with active HHV-6 and/or HHV-7 infection on admission to and discharge from hospital (Table 2).

Table 2. Dynamics registration of the herpesviruses infection in the patients

Period of the study	Patients (n)	HHV-6		HHV-7		HHV-5 (CMV) viremia		HHV-4 (EBV) viremia	
		viremia	latent	viremia	latent	+ HHV -6	+ HHV -7	+ HHV -6	+ HHV -7
admission to hospital	21	10	12	5	7	9	5	10	5
discharge from hospital	17	4	10	3	7	1	0	2	1
period of reconvalescence	5	1	5	1	5	ni*	ni	ni	ni

* not investigated

There was carried out examination of 21 patients in the sharp phase of infection and 17 patients after the course of therapy. While in hospital the patients received the antiviral therapy, immunotherapy and symptomatic therapy. The results of the conducted examinations showed that active HHV-6 infection was recorded in 10 patients on admission to hospital and in 4 patients after discharge from hospital.

The latent form of infection was detected in 12 patients when admitted to hospital and in 10 patients after discharge from hospital. HHV-7 plasma viremia was discovered in 5 patients and latent/persistent infection – in 7 patients after the hospital treatment. The active form of the infection remained in 2 and latent/persistent – in 7 patients after the course of treatment.

Concurrent viral infection was detected practically in all (9 out of 10) patients. CMV was detected alongside with HHV-6 and in 10 patients – EBV infection. HHV-7 infection was found in all patients with CMV and EBV infection.

Two patients (No 1 and No 2), hospitalized with fever of unclear etiology as the primary diagnosis, had prolonged viremia and some of the basic clinical signs of CFS (Table 3). This investigation allows diagnosing CFS for patients No 1 and No 2.

Table 3. Registration of HHV-6 and HHV-7 infection markers and clinical symptoms of the chronic fatigue syndrome.

Markers of viruses infection and clinical symptoms	admission to hospital		discharge from hospital	
	№1	№2	№1	№2
HHV-6 DNA in PBL/Plasma DNA	–/+	+/+	+/+	+/–
HHV-7 DNA in PBL/Plasma DNA	+/–	–/–	+/	–/–
Increased fatigue	+	+	+	+
Increased fatigue (6 months and more)	+	+	+	–
Subfebrile temperature during 6 and more months	+	+	+	–
Sleep disturbances	+	+	+	+
Emotional lability	+	+	+	+
Headache	+	–	+	+

CONCLUSION

The conducted research revealed the frequency of HHV-6 and HHV-7 occurrence in patients with different pathology; the greatest percentage was recorded in patients with chronic infections and with diseases of the central nervous system.

Active HHV-6 and HHV-7 infections were recorded in the patients whose diagnose was fever of unclear etiology. Active HHV-6 infection was recorded almost in a half of the patients (47%) with chronic HHV infection. Long-term viremia and some of the main clinical signals were registered in two patients whose primary diagnosis was fever of unclear etiology.

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ASSOCIATION OF HHV-6 AND HHV-7 WITH DISEASES OF THYROID GLAND

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INTRODUCTION

Diseases of the thyroid are manifested by qualitative or quantitative alterations in hormone secretion: insufficient hormone secretion – hypothyroidism or excessive secretion of hormone – hyperthyroidism or thyreotoxicosis; enlargement of the thyroid (goiter) – generalized or focal (nodular). Enlargement of the thyroid gland may be associated with increased, normal or decreased hormone secretion, depending upon the underlying disturbance. Focal enlargement usually reflects neoplastic disease.

Hashimoto's thyroiditis (lymphadenoid goiter) is a common chronic inflammatory disease of the thyroid in which autoimmune factors play a prominent role. Evidence of the participation of autoimmune factors includes the lymphocytic infiltration of the gland and the presence of increased concentrations of immunoglobulins and of antibodies against several components of the thyroid tissue in the serum. This disorder coexists also with some other diseases of an autoimmune nature, like Grave's disease.

Unfortunately, there is no precise statistics about how many people in Latvia suffer from thyroid gland disorders, but in the USA these problems are afflicting up to 10% of the population [Canaris G. J. et al., 2000]. Thyroid gland autoimmune disorders, mostly the autoimmune chronic lymphoid thyroditis, present an increasing problem in the Northern part of Europe, Latvia including. The term thyroiditis encompasses a heterogeneous group of disorders characterized by some form of thyroid inflammation.

Viral infections are frequently cited as a major environmental factor implicated in subacute thyroiditis and autoimmune thyroid diseases (AITD) [Prummel M., et al., 2004]. Viral persistence and inflammation can act synergistically also to induce and sustain autoimmunity by either unveiling cryptic self-epitopes or favouring determinant spreading, or activating dendritic cells, or promoting constant priming of new autoreactive T-cells, or to contributing to the efficient generation of effector cells, or restimulating memory T-lymphocytes. Inflammatory and autoimmune processes with unknown etiology often form the background for diseases of the thyroid gland as well. Direct evidence of the presence of viruses or their components in the thyroid gland are available for retroviruses (HFV) and mumps in subacute thyroiditis, for retroviruses (HTLV-1, HFV, HIV and SV40) in Graves's disease and for HTLV-1, enterovirus, rubella, mumps virus, HSV, EBV and parvovirus in Hashimoto's thyroiditis [Kawai H., et al., 1996; Vrbikova J., et al., 1996; Nakachi K., et al., 1997; Mitteldorf C. A., et al., 1999; Espino Montoro A., et al., 2000; Parmar RC et. al, 2001; Mori K., et al., 2007]. During the last few years much attention has been paid to the members of the *Herpesviridae* family demonstrating that they are involved in the development of inflammatory and autoimmune diseases. Human herpesviruses (HHVs) are ubiquitous with widespread tissue tropism and have been found in the thyroid, which can be a reservoir of latent HHVs [Chen T. & Hundall S. D., 2006]. However, the association of HHVs with inflammatory and autoimmune diseases of the thyroid gland has been investigated insufficiently so far.

The aim of this work was to clarify if there is any association between latent/persistent herpesviruses (Human Herpesvirus 6 [HHV-6] and Human Herpesvirus 7 [HHV-7]) infection and diseases of the thyroid gland in Latvia.

MATERIALS AND METHODS

The Ethics Committee of Riga Stradins University, Latvia, approved the study and informed mutual consent was obtained from a total of 42 subjects (37 females, 5 males, 23 to 77 years old, mean age 50 years) that were consecutively referred to thyroidectomy because of the thyroid gland pathology without defining a more precise diagnosis before the examination. The exact diagnosis of the thyroid gland pathology was established by histological analysis of resected tissue and analysis of thyroid gland hormonal activity.

Groups of patients: 1st group (n=9): patients with *Struma nodosa III Eutireoticum* without autoimmune process; 2nd group (n=7): patients with *Struma nodosa III Eutireoticum* with autoimmune process; 3rd group (n=17): patients with *Struma nodosa III Thyreotoxicum* without autoimmune process; 4th group (n=5): patients with *Struma III nodosa Thyreotoxicum* with autoimmune process; 5th group (n=4): patients with papillar cancer of the thyroid gland.

EDTA blood, plasma and resected tissue were collected from each patient, aliquoted and stored at -70^0. DNA was isolated from whole blood and restricted tissues using phenol-chloroform method. QIAamp DNA Blood Mini Kit was used to extract DNA from plasma. β-globin PCR was applied in order to check DNA quality. Negative β-globin PCR result for DNA isolated from plasma shows that there is no cell DNA in the sample. This is very important for virus reactivation detection. Nested polymerase chain reaction (nPCR) with the corresponding primer pairs were used for the detection of HHV-6 and HHV-7 genomic sequences [Bandobashi, et al. 1997; Berneman, et al., 1992, respectively] in DNA isolated from whole blood, resected tissue and plasma. To exclude the possibility of contamination during the PCR, HHV-6 and HHV-7 negative DNA, water controls were included in each experiment as well. The amplification products were visualized in 1.7% agaroze gel with ethidium bromide staining and analyzed using Kodak Electrophoresis Documentation and Analysis System (EDAS) 290.

RESULTS

HHV-6

2/9 (22.2%) patients from the 1st group had HHV-6 genomic sequence in blood DNA only. There were no patients from other groups who carried this sequence in blood DNA only. In tissue DNA only 3/9 (33.3%) patients from the 1st group, one patient (1/7; 14.3%) from the 2nd group, 9/17 (52.9%) patients from the 3rd group, 3/5 (30.0%) and 3/4 (75.0%) patients from the 4th and the 5th groups respectively had HHV-6 DNA genomic sequence. Simultaneously in blood and tissue DNAs HHV-6 genomic sequence was detected in all examined groups without statistically significant difference. However, in the patients of the 2nd group it was revealed more frequently (4/7 (57.1%)) in comparison to other groups: 4/9 (44.4%) patients in the 1st group, 3/17 (17.6%) patients in the 3rd group, 2/5 (40.0%) patients in the 4th group and in one (1/4; 25.0%) patient in the 5th group. HHV-6 specific sequence in blood, tissue and plasma simultaneously was not revealed in three out of five examined groups (1st, 4th and 5th), but in the 2nd and in the 3rd group HHV-6 genomic sequence was detected in one (1/7; 14.3%) and in 4/17 (23.5%) patients respectively. None of the examined patients had HHV-6 genomic sequence in blood and plasma DNA simultaneously and two patients – one from the 2nd group (1/7; 14.3%) and one from the 3rd group (1/17; 5.9%) – were without HHV-6 genomic sequence (Figure 1).

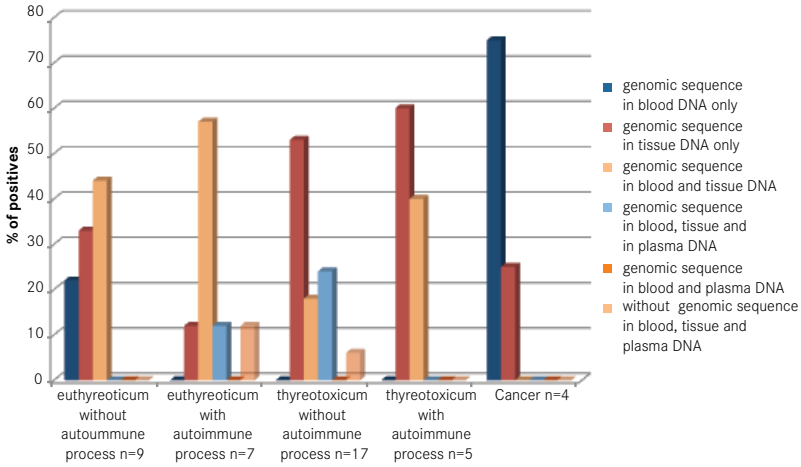

Figure 1 HHV-6 prevalence in patients with Struma nodosa III euthyreoticum and thyreotoxicum (without and with autoimmune process or cancer)

HHV-7

HHV-7 genomic sequence in blood DNA only was detected in three from the examined five groups: in one (1/7; 14.3%) patient from the 2nd group, in 4/17 (23.5%) patients from the 3rd group and in one (1/4; 25.0%) patient from the 5th group. Virus specific sequence in tissue DNA only was prevalent in the 2nd and the 3rd group (2/7; 28.6% and 1/17; 5.9% respectively). HHV-7 genomic sequence in blood and tissue DNAs simultaneously was found in all groups except the 5th: 3/9 (33.3%) in the 1st, 2/7 (28.6%) in the 2nd, 6/17 (35.3%) patients in the 3rd and in one (1/5; 20.0%) patient from the 4th group (Figure 2).

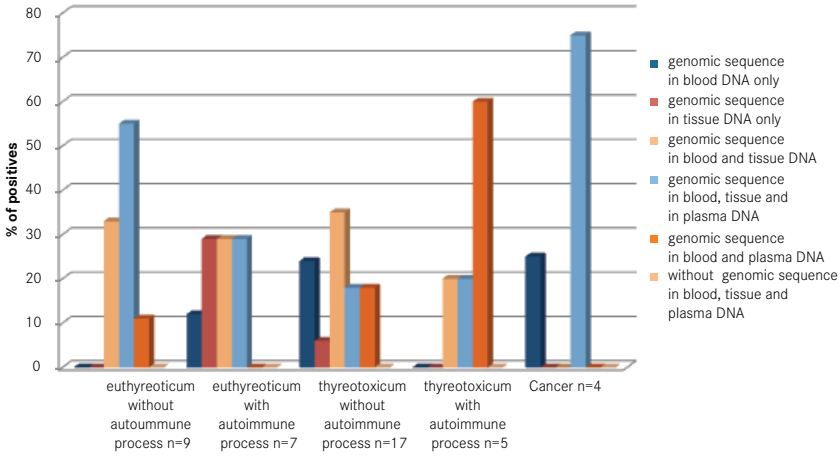

Figure 2 HHV-7 prevalence in patients with Struma nodosa III euthyreoticum and thyreotoxicum (without and with autoimmune process or cancer)

Simultaneous HHV-7 genomic sequence in blood, tissue and plasma DNAs was observed more frequently in the 5th group (3/4; 75.0%) in comparison with the other examined patients: in 5/9 (55.5%) patients from the 1st group, in 2/7 (28.6%) patients from the 2nd group, in 3/17 (17.6%) patients from the 3rd group and in one (1/5; 20.0%) patient from the 4th group. Genomic sequence in blood and plasma DNAs simultaneously was found in 3 patients from the 3rd and the 4th group (3/17; 18.0% and 3/5; 60.0% respectively) and in one (1/9; 11.1%) patient from the 1st group. There was no patient in the examined groups without HHV-7 genomic sequence in any of the specimens examined. (Figure 2). Statistically significant difference was not detected between HHV-7 prevalence between different DNAs or the groups investigated.

DISCUSSION

The β-herpesviruses infection in the human population is widely spread throughout the world, including Latvia.

The data provided are preliminary, more serious work with mRNA extraction, viral load detection, detection of antiviral antibodies, TNF-α, IL-6, IL-1β expression level and epigenetic analysis of TSHR, TTF1, TTF2, ERp29, APC, RAR-β, PAX8 and TSHR genes is in progress, nevertheless our nPCR data show that HHV-6 and/or HHV-7 prevalence in the thyroid gland tissues is high (95.2% examined thyroid tissue specimens carried genomic sequences of herpesviruses). The patient groups are too small yet to analyze differences between them and an impact of viruses, but it is possible to see cetain trends. HHV-6 and HHV-7 genomic sequences, especially in the thyroid gland tissue DNAs are common in all examined groups and there is no statistical difference between the freequency of HHV-6 and/or HHV-7. The fact that in the 1st, 4th, and 5th group there was no HHV-6 genomic sequence in blood plasma indicates that the virus is in a latent stage, however, without mRNA extraction that shows an early stage of replication of the virus or at least IgM tests and/or elevated IgG detection we cannot be 100% sure about it. Without extraction of mRNA from tissue, we cannot tell if there is ongoing active HHV-6 and/or HHV-7 infection. A researcher group from the USA did anatomical mapping of human herpesviruses, they observed HHV-6 but not HHV-7 DNA sequences in the thyroid gland tissues [Tiansheng Chen and S David Hudnall, 2006]. Likewise in our study we can find more frequently HHV-6 genomic sequence (85.7%) in comparison with HHV-7 (57.4%) genomic sequence in the thyroid gland tissue. There are only a few data about HHV-6 and HHV-7 association with autoimmune thyroid gland disorders in literature, in one of them Leite and co-authors analyze these two viruses and Graves disease (GD). Their results showed that HHV-6 infection rates were similar in GD patients and among the control group. HHV-7 infection increased the risk of developing GD by more than three times [Leite, et al., 2010]. In our laboratory a few years ago we did a small pilot study on lymphotropic herpesviruses infection and diseases of the thyroid gland. This work also confirmed a high prevalence of HHV-7 in the thyroid glan tissues [Murovska, et al., 2003]. It is imporant to remember that HHV-6 and HHV-7 are lymphotropic viruses and latent/persistent infection of those viruses in healthy population is high. In a case of chronic inflammatory process lymphocyte infiltration has been observed and findings of HHV-6 and HHV-7 genomic sequences can be explained by the presence of HHV-6 and/or HHV-7 carrying lymhocytes in the thyroid gland tissue. To be able to draw final conclusions about the possible HHV-6 and/or HHV-7 role in ethiopatogenesis of the thyroid gland autoimmune and non-autoimmune disorders, more serious research needs to be done.

PRELIMINARY CONCLUSION

Lymphotropic HHV-6 and HHV-7 prevalence in the thyroid gland tissues in patients with different thyroid diseases is high. However, it remains to establish whether they are responsible for thyroid diseases or whether they are just innocent bystanders.

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USAGE OF NATURAL GLYCOPEPTIDE FOR TREATMENT OF HERPESVIRUSES INFECTION

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INTRODUCTION

Immunomodulators from natural sources nowadays play an important role in treatment of different diseases. The medical properties of lactic acid bacteria have been in the focus of attention since the end of the 18th century. The health benefits of the friendly bacteria first came to the attention, when Dr. I. Metchnikoff, a Russian biologist, recognized that certain white blood cells, known as phagocytes, ingest and destroy dangerous bacteria. Later Dr. Jules Freund, an internationally known immunologist, invented a preparation consisting of the cell wall of lactic acid bacteria which stimulated a rapid reaction of the immune system named Freund's adjuvant. However, the exact substance within the cell wall, which influenced the immune system, remained unknown. The research continued until finally the substance was identified. The substances of muramyl-dipeptide chain, in particular glucosoaminnylmuramyl dipeptide (also called disaccharide-dipeptide or GMDP), are responsible for stimulating of the immune system (1).

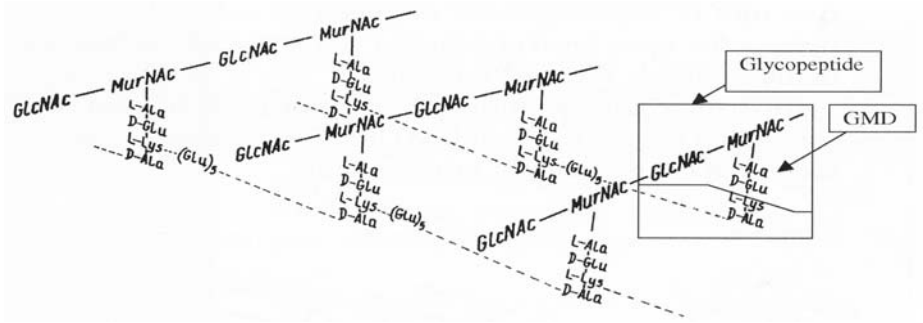

Figure 1. The net structure of glycopeptides and GMDP

Glicopeptides are the polymer components of the cell wall of almost all procariotic organisms (2). They represent heteropolymers consisting of long glycan's chains connected by diametrical –short peptide's bridges. Peptides, which carry diametrical links between glycan's chains, are built mostly from L and D-alanine, dicarbone acids (D-glutamine and D-asparagine). The presence of D-aminoacids in the structure of biopolymers is very rare in nature, but it is very typical in all peptidoglycans of the cell wall. The link between separate peptide's chains is realized by cross-peptide bridges, built from neutral amino acids – for lactobacillus it is D-isoasparagine. GMDP (Disaccharide-Dipeptide) is the main structural element of peptidoglycan of the cell wall of all gram-positive bacteria, including almost all lactic acid bacteria. The stability of the cell wall of St. aureus is based on the compact structure of peptidoglycan. If its reactive groups are removed all carboxyl groups of muramic acid are linked to polypeptide's chains, which are connected to each other by cross bridges. The cell walls provide a means to influence lysozyme and are used as substrate for detecting the activity of this enzyme. Sensitivity of this organism to lyzozyme could be explained by the structure of peptidoglycan and its cell

wall: about half of which is muramic acid and is not linked to peptide's chains. The link between the main peptide's chains is attached directly to aminoacids (3).

MATERIALS AND METHODS

Studying the composition of lyzozyme hydrolysate of the cell walls of *Lactobacillus bulgaricus*, Bulgarian scientists have determined that glycopeptides possess antiviral and antitumour activities. On the basis of this a hydrolysate has been developed, the medicine called Blastolysin. At the same time research developed in the area of synthesis of glycopeptides. The original method of synthesis of glycopeptides consists of the condensation of unprotected disaccharide from *Micrococcus lisodeitikus* with synthetic dipeptide. The production technology of GMDP, based on this method, was realised by the Biolar company Olaine, Latvia, in 1986. Pre-clinical trials of GMDP and the development of drug forms were realized by the experimental plant of the Institute of Organic Synthesis (currently Grindex). Since 1991 the Peptech company has been developing the biotechnological production of GMDP, using the technological equipment at Shemjakin's and Ovchinikov's Institute of Bioorganic Chemistry at the Russian Academy of Sciences. The commercial name of this medicine is Licopid (4, 5).

In the authors' opinion GMDP is capable of stimulating a specific immune response by activating macrophages, which in their turn activate T and B-lymphocytes; the latter being associated with the existence of specific receptors whithin the cell.

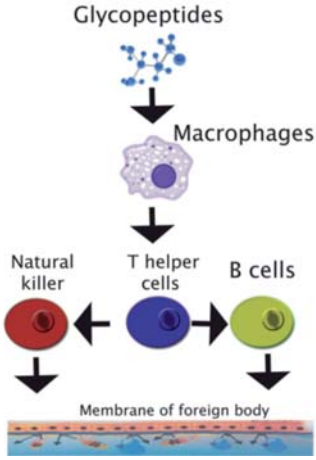

Figure 2. Mechanism of immune response induced by glycopeptides.

Phagocytic cells (neutrophils and macrophages) secrete cytokines (IL- 1, IL- 6, TNF – alfa and others), activate T and B-lymphocytes and stimulate the specific immune response (4,5).

The rapid development of the production technology of lactic acid bacteria and its use as a food supplement for prevention of various diseases created a growing interest in the development of effective methods of isolation of natural glycopeptides from lactic acid bacteria. This is the reason why a group of Latvian scientists have developed a unique technology of isolation and purification of natural soluble glycopeptides, containing as the main structural unit disaccharide-dipeptide from the lactic acid bacteria's cell wall, by using natural food grade starting materials only (2). Scientific research and experiments show that during phagocytosis of grampositive bacteria (lactic acid bacteria) the enzymes of macrophages split peptidoglucans of such bacteria and form glycopeptides, containing disaccharide-dipeptide, with following secretion of glycopeptides in the environment. It has been proven that glycopeptides are

constantly delivered from the gastro-intestinal tract into the body environment and they are natural regulators of the immunity. The presence of glycopeptides was found in mother's milk and such products as yoghurts, kefir and different probiotics that are recognized to be very healthy. If stimulation of the immune system can be achieved with the help of live probiotic bacteria, why use natural glycopeptides? The answer is very simple: for probiotic bacteria to influence and stimulate the immune system it has to be absorbed and split by macrophages, thus producing glycopeptides. This means that macrophages must have a full set of well functioning enzymes. In case of the immune system disorders the functional activity of macrophages is very low, leading to a very low ability to split peptidoglucans and produce glycopeptides containing disaccharide-dipeptide. Under such circumstances the whole intact live bacteria will be less effective or even ineffective. This is why it is essential to deliver already prepared soluble glycopeptides into the blood stream and stimulate the immune system.

Glycopeptides have been evaluated in many studies and have been found to have no side effects, no toxicity and no drug interractions. Glycopeptides were prepared in our laboratory and their quality was tested for citotoxicity in tissue culture cells A 549 during 24 to 72 hours (6).

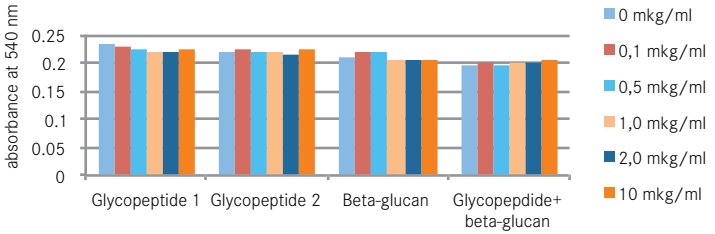

Figure 3. Detection of citotoxicity of different natural immunomodulators in A 549 cell culture during 24 hours.

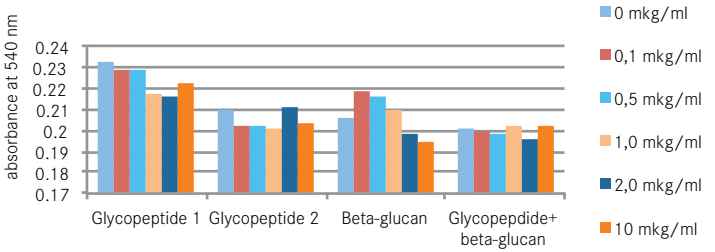

Figure 4. Citotoxicity detection of different natural immunomodulators in A 549 cell culture during 48 hours.

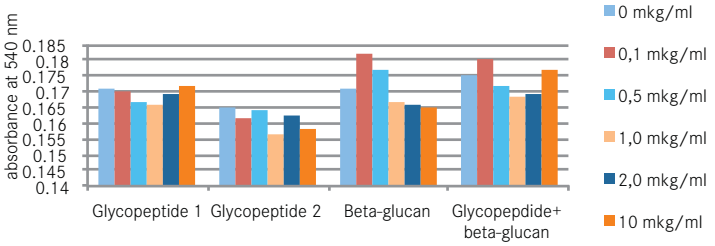

Figure 5. Citotoxicity detection of different natural immunomodulators in A 549 cell culture during 72 hours.

DISCUSSION

A very important property of natural glycopeptides is its ability to stimulate leucopoesis (7). The different glycopeptide compositions together with other immunomodulators are used in medicine for prevention and treatment. Considering all the above mentioned properties, glycopeptides together with beta-glucans and other immunomodulators are used for immune correction as a component of a complex therapy of various infections associated with the immune suppressed status, including:

- chronic viral hepatites
- cytomegalovirus or Herpesvirus 5
- tuberculosis
- tick encephalitis
- acute and chronic purulent processes and inflammatory lung diseases
- in oncology during chemotherapy and X-ray therapy (8)
- different sexually transmitted diseases

RESULTS

Good results were obtained, if glycopeptide compositions were used together with medicines, mainly antibiotics. In this case drugs were more effective and a shorter time was neccessary for patient treatment. Clinical observation was performed on hepatitis C, because it is one of the most critical and dangerous diseases. The clinical trial "Efficiency of the Glycomun (glycopeptide) food supplement in the treatment of viral hepatitis C" was carried out in the State agency Infectology Centre of Latvia together with Rīga Stradiņš University. The results of the trial were satisfactory. The following changes in the parameters were traced in the trial group in comparison with the control group: increase of the mean erytrocyte volume and normalisation of the said parameter, normalisation of platelet level, increase of platelet anisocytosis, increase of the absolute number of neutrophils; increase of the percentage of neutrophils; normalisation of the relative number of lymphocytes, increased level of reduced glutatione; increase of absolute number and percentage of activated T-lymphocytes, hemoglobin level and SPGOT. There was detected a clinically significant improvement of the life quality indicators – the "Emotional impact'' scale and the "Overall mental health''scale indicators. The tendencies toward changes in the biochemical and quality indicators, which were observed in the trial group, may be ascribed to the effects of the medicine. The significance (reliability) of the results is diminished by the small size of the groups, heterogeneity of the studied groups in terms of biochemical indicators (absolute number and relative quantity of mixed group cells, platelet anisocytosis, percentage of neutrophils) and the short trial period (9).

Clinical observation was done only for three patients with the CMV infection. Human cytomegalovirus (CMV) is ubiquitous. The virus has infected most individuals by early adulthood in the developing countries. Most individuals will show no symptoms as a result of either primary infection, reactivation, or reinfection, manifesting that the virus has been well adapted to its normal host. CMV is a member of the *Betaherpesvirinae*, subfamily of the *Herpesviridae*. This classification was originally based on its slow growth in vitro and strict species specificity and now is based on the genetic sequence homologies among the alpha, beta and gamma subfamilies. These genetic diferences do not allow classification into district genotypes. Strains are still the best characterized as having an antigenic mosaic, which is recognized broadly by the host cellular and humoral immune responses. Individuals infected with one strain of CMV have cross-reactive immunity against all strains. Strains of CMV are resistent to some drugs, e. g., ganciclovir a. o., and this was the reason to start treatment with glicopeptides. Certain results were seen already after the first three months as the patients felt better and the life quality indicators went up. Viral load was not performed because the patients underwent ambulatory treatment (10).

CONCLUSION

A new family of biologically active natural immunomodulators could be perspective in medicine. A combination of immunomodulators from natural sources lactobacillus, yeasts, medicinal mushrooms and other glycopeptides together with β-glucans and other biologically active compounds and a combination with antibiotics and drugs can be succesfully used for treatment and prevention for many diseases: different cancers, HIV, hepatitis C and B, chronic heart diseases, tuberculosis, sexually transmitted diseases, pneumonia, different herpesviruses infections and other diseases.

ACKNOWLEDGEMENTS

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ANTIVIRAL AGENTS ACTIVE AGAINST HHV-6 AND HHV-7

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OVERVIEW

The aim of this short presentation is not to give a historical overview of the antiviral drug development or an analysis of this process, but to outline the current situation. At the same time at the very start it is important to stress that the term “antiviral agent” does not mean a patient’s treatment or therapy of infectious disease, which is a complex process by itself, depending on the disease manifestations.

Although public health measures and vaccines are the most effective ways for controlling many viral infections, preventive measures have not succeeded with numerous viral diseases. Antiviral drugs have been developed for some

of these diseases. Despite the success, there are still relatively few diseases for which highly effective antiviral drugs have been developed.

The first antiviral drugs were discovered by testing chemicals for their ability to inhibit virus replication, a more modern and rational approach was target- or cell-based throughput screenings, and the latest rational approach is to use detailed knowledge about a viral reproduction cycle to design drugs that will inhibit its activity (1). In principle, any stage of viral reproduction can be targeted for inhibition. There are potential advantages to targeting very early or late stages such as attachment, entry, and release, because inhibitors of these steps do not have to enter cells to exert activity. Such stages as genome replication, assembly, and maturation often require specific viral enzymes which are attractive drug targets: most antiviral drugs currently available inhibit genome replication (2), see the latest updates in **Table 1** (3). A major advantage of the antiviral drug development is that quantitative reduction in virus yield is a measure of activity and correlate with the clinical picture. Selectivity and mechanism of antiviral action are also crucial for the clinical use of antiviral drugs. At the same time, the best way to understand antiviral specifity and mechanism of action is through the study of drug resistance. As viruses are obligate intracellular parasites, the detection of resistance to an antiviral drug implies that the drug is selective (4).

The biggest part of antiviral drugs inhibits viral genome replication, and nearly all of them inhibit a DNA polymerase. Those viruses whose polymerases have been successfully targeted include certain **Human Herpesviruses**

Most of these drugs are nucleoside analogues (2, 3, 4). All nucleoside analogues must be activated by phosohorylation, usually to the triphosphate form, to exert their effect. Phosphorylated nucleoside analogues inhibit polymerases by competing with the natural dNTP substrate; they are usually incorporated into the growing DNA chain, where they often terminate elongation. Either or both of these features – inhibition and incorporation – can be important for antiviral activity (2, 4).

The various human herpesviruses encode both – kinases and DNA polymerases. These enzymes differ sufficiently from their cellular counterparts to permit the development of selective antiviral nucleoside analogues. A number of antiviral nucleoside analogues, including vidarabine, idoxuridine, and trifluridine, were developed and used against HSV infections (1, 5). However, these drugs have been superseded by more selective compounds.

Table 1. Antiviral Drugs

Drug	Viruses	Chemical Type	Target
Vidarabine	Herpesviruses	Nucleoside analogue	Virus polymerase
Acyclovir	Herpes simplex (HSV)	Nucleoside analogue	Virus polymerase
Gancyclovir and Valcyte ™ (valganciclovir)	Cytomegalovirus (CMV)	Nucleoside analogue	Virus polymerase (needs virus UL98 kinase for activation)
Nucleoside-analog reverse transcriptase inhibitors (NRTI): AZT (Zidovudine), ddI (Didanosine), ddC (Zalcitabine), d4T (Stavudine), 3TC (Lamivudine)	Retroviruses (HIV)	Nucleoside analogue	Reverse transcriptase
Non-nucleoside reverse transcriptase inhibitors (NNRTI): Nevirapine, Delavirdine	Retroviruses (HIV)	Nucleoside analogue	Reverse transcriptase
Protease Inhibitors: Saquinavir, Ritonavir, Indinavir, Nelfinavir	HIV	Peptide analogue	HIV protease

Drug	Viruses	Chemical Type	Target
Ribavirin	Broad spectrum: HCV, HSV, measles, mumps, Lassa fever	Triazole carboxamide	RNA mutagen
Amantadine/Rimantadine	Influenza A strains	Tricyclic amine	Matrix protein/ haemagglutinin
Relenza and Tamiflu	Influenza A and B strains	Neuraminic acid mimetic	Neuraminidase Inhibitor
Pleconaril	Picornaviruses	Small cyclic	Blocks attachment and uncoating
Interferons	Hepatitis B and C	Protein	Cell defense proteins activated

SUMMARY OF ANTIVIRAL AGENTS:

Ganciclovir CYTOVENE™

Ganciclovir is a drug used to treat CMV retinitis. It is available in both oral and intravenous forms. Studies have shown that **HHV-6** replication is effectively suppressed by intravenous ganciclovir and the drug has been used to successfully treat life-threatening HHV-6 infections of the brain and the spinal cord in bone marrow transplant recipients. Ganciclovir is the only drug that has demonstrated its ability to successfully treat brain infections by **HHV-6.** Treatment with intravenous ganciclovir may cause potentially serious side effects, most commonly bone marrow suppression. Oral ganciclovir is available, but it produces relatively low serum levels of the drug and is unlikely to be highly effective against rhe established HHV-6 infections.

Valganciclovir VALCYTE™. Recently, the valine ester of ganciclovir (valganciclovir or VALCYTETM) has been developed as an antiviral drug, and it was recently FDA approved for use in the treatment of cytomegalovirus retinitis in patients with AIDS. Valganciclovir is a pro-drug of ganciclovir as it is rapidly converted to ganciclovir by intestinal and hepatic enzymes producing plasma levels of a drug that are similar or even superior to those achieved by intravenous ganciclovir. Valganciclovir is administered orally twice per day.

Beta Interferon AVONEX™ or BETASERON™. Interferons are used to treat certain types of cancer, chronic infections (e. g., Hepatitis C) and other diseases of infectious or autoimmune origin, such as multiple sclerosis. Interferon is also known for antiviral properties. Various laboratory studies have shown that strains of **HHV-6** are sensitive to suppression by beta interferon. This finding is consistent with the known antiviral activity of interferon.

Acyclovir ZOVIRAX™. Acyclovir is used to treat herpes simplex (HSV), varicella zoster (VZV) infections. It is available in oral form. The available data indicate that **HHV-6** and **HHV-7** is relatively insensitive to the inhibitory effects of acyclovir. The mean inhibitory concentration 50% (IC50) of acyclovir for HHV-6 strains is approximately 30 μM, a concentration well above the plasma levels achievable with either oral or intravenous therapy.

Acyclovir VALTREX™. Valacyclovir or VALTREX is an orally delivered drug chiefly used to treat HSV and VZV. It is a pro-drug of acylovir, meaning that it is converted to active acyclovir within the body. This results in higher levels of drug in the blood stream and it is believed that this level of drug might be partially effective against **HHV-6** and **HHV-**7. Valcyclovir has been used to effectively decrease the incidence of **HHV-6** associated disease in bone marrow transplant recipients. Thus, it is effective against reactivation of **HHV-6**, but may not be effective in suppressing an active, chronic infection. Studies have also demonstrated that VALTREX therapy at standard dosages is associated with a low rate of adverse side effects. Thus, VALTREX treatment stands as a potential alternative for long-term therapy for **HHV-6** associated diseases, especially in combination with other antiviral drugs such, as beta interferon.

Foscarnet FOSCAVIR™. Foscarnet is used to treat CMV retinitis. It is available in injectable form. Literature concerning the sensitivity of **HHV-6** and **HHV-7** replication to suppression by foscarnet is quite consistent in stating that all virus strains tested showed marked sensitivity to the drug. However, treatment with intravenous foscarnet carries with it a significant risk of toxicity, which most commonly manifests as renal dysfunction and electrolyte imbalances.

Cidofovir VISTIDETM or (S)-HPMPC. Cidofovir is used to treat CMV retinitis in patients with AIDS. Intravenous administration of cidofovir can be associated with significant renal toxicity, although it appears to be less toxic than either foscarnet or gancyclovir. Cidofovir is available for use in off-label applications, such as the treatment of **HHV-6** associated disease. Two cell culture based studies have reported that cidofovir can effectively suppress the replication of **HHV-6** and **HHV-7**, although this observation has not been confirmed by other investigators.

Nonconventional Antiviral Agents. Several preparations of various types have been assessed for their ability to suppress the replication of **HHV-6** and HHV-7 in cell culture. The potential of these agents to be used in a clinical setting remains unclear and little or nothing is known concerning their pharmokinetics or the plasma levels they can achieve.

As one can see, ganciclovir, foscarnet and cidofovir have been reported to be inhibitors of **HHV-6** and **HHV-7** replication in vitro, although not consistently (). There have been no controlled trials of antiviral therapy against **HHV-6** and **HHV-7** infection, but individual published cases suggested a clinical response to the treatment of **HHV-6** disease after bone marrow transplantation, using either ganciclovir or foscarnet, or both (6, 7, 8, 9).

CONCLUSION

The currently licensed anti-herpetic compounds may be effective against **HHV-6** and **HHV-7**, but treatment strategies need to be formulated through appropriate clinical protocols (6): ganciclovir, phosphonoformate (foscarnet) and cidofovir are potent inhibitors of HHV-6 and HHV-7 replication *in vitro*; acyclovir (ACV) and other thymidinkinase-dependent drugs are marginally effective. The sensitivity of HHV-7 to the guanine analogs was different from HHV-6, suggesting a difference in selectivity of specific viral enzymes (7).

As with other herpesviruses, HHV-6 and HHV-7 establish latent infections in monocyte-macrophages and CD4+ T lymphocyte, respectively. The mechanisms by which latency is established and reactivated are not yet known, but it is out of the question that using of virus specific antivirals in this stage does not cure the disease. The future use of immunotherapeutic approaches may complement the current management strategies.

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