PhD Thesis

Seroepidemiology of human herpesvirus-8 infection in Hungary

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Introduction:

Taxonomy, structure and genomic organisation of HHV-8

The hypothesis of the putative infectious etiologic agent of Kaposi's sarcoma was proven in late 1994. Representational difference analysis (RDA) was used to identify the agent of KS. Using this molecular biologic method DNA samples from KS and healthy tissues were compared, and unique herpesvirus-like sequences were identified in the KS tissue. These sequences appeared to define a new human herpesvirus named KS-associated herpesvirus (KSHV) or human herpesvirus type 8 (HHV-8) and it was proposed that this may play a role in the etiology of these tumour.

The members of the herpesvirus family are all structurally similar. They are linear double-stranded DNA viruses that are enveloped. Human herpesvirus-8 is a gamma-2 herpesvirus (rhadinovirus) with the size of approximately 140 nm.

The HHV-8 genome consists of a single continuous long unique region of 140-kb containing all identified coding regions. The long unique region is flanked on both sides by terminal repeats (TRs) consisting of several 801 bp repeat subunits that are predominantly of G+C content. The unique region of the genome contains approximately 80 putative open reading frames (ORFs) encoding for the enzymes and viral structural proteins found in the herpesviruses, but also contains a unique number of ORFs that were pirated during viral evolution from cellular genes. These include proteins that alter cellular growth, induce angiogenesis and regulate antiviral immunity. The unique KSHV genes without homology to genes identified to date were assigned the 'K' prefix and numbered sequentially.

The role of HHV-8 in the pathognesis of KS

Kaposi's sarcoma (KS) is a vascular type of malignant neoplasm. It appears to arise de novo from endothelial cells that line lymphatic or blood vessels in the skin or visceral organs. It is distinguished from other vascular tumours in that it involves the development of multiple cutaneous lesions with occasional local invasion of the underlying tissues. The morphology of KS ranges from the early stages of patches to raised plaques and at a later stage the lesions become nodular. KS has been classified into 4 different epidemiologic forms referred to as

classic KS, endemic KS, KS in immunosuppressed patients, and epidemic KS. Histologically the KS lesions are characterised by atypical neoangiogenesis, spindle cells and an inflammatory cell infiltrate. The spindle cells are thought to be the neoplastic component of the lesions.

Using PCR technique, HHV-8 can be detected virtually in all KS tissue samples irrespective of the clinical stage or the epidemiological form of the KS. In situ techniques were used to localise HHV-8 DNA or RNA within the KS tissue. Viral nucleic acids were detected in most of the spindle cells and atypical endothelial cells lining distended vessels and in some of the tumour infiltrating macrophages or inflammatory cells. The lesions predominantly contained latently infected spindle cells that were found to have multiple copies of the circularised KSHV DNA per cell. Latent transcripts that were detected in KS include ORF 73 which encodes LANA, ORF 72 which encodes v-cyclin, ORF 71 which encodes v-FLIP and ORF K12 which encodes kaposin. These KSHV specific transcripts are transcribed during viral latency and their products may play a role in KSHV driven oncogenesis. Approximately 2-5% of spindle cells and some of the tumour infiltrating monocytes enter the lytic cycle and are characterised by lytic RNA transcripts. Lytic transcripts like v-GPCR and v-MIPs have importance in KSHV driven atypical neoangiogensis.

In the following chapters, the function of ORFs that are thought to contribute to the pathogenesis of KS will be discussed.

ORF 73/LANA

Cells harbouring latent HHV-8/KSHV infection have a tricystronic transcript from ORF 73-72-71. These cells express a high molecular weight (222-234 kDa) latent nuclear antigen doublet also called as LANA from ORF73. Latently infected cells have multiple copies of circularised KSHV DNA which are maintained as episomes. Viral DNA was found to be associated with the chromatin in the interphase nucleus and were associated at random sites of the metaphase chromosome by FISH (fluorescence in situ hybridisation). According to simultaneous LANA immunofluorescence staining and HHV-8 specific FISH it was shown that LANA dots co-localise with KSHV episomes in the nuclei of KSHV infected cells. The association of both LANA and KSHV with chromosomes indicates that LANA has a function in maintaining KSHV DNA in infected cells. In addition, the complex of LANA and KSHV

DNA on the chromosomes would ensure an efficient distribution of viral episomes to progeny cells and the inclusion of KSHV DNA in newly formed nuclei. The finding that LANA is restricted to sites of KSHV DNA in interphase nuclei is indicative of specific recognition of KSHV DNA by LANA. The repetitive TR DNA was determined as the specific DNA recognised by LANA within the HHV-8 genome. It was also shown that LANA interacts with histone H1. LANA expressing cells were able to maintain episomal HHV-8 fragments containing several TR units and few hundred bps from the leftmost end of the HHV-8 genome. This provides further evidence that the function of LANA is to allow the episomal persistence/replication of the KSHV genome in nuclei by binding to histone H1 and to the origin of viral episomal replication.

Many of the tumour viruses have been shown to inhibit the anti-oncogene (tumour suppressor gene) function in cells allowing for cellular proliferation and tumourigenesis to occur. Tumour suppressor protein p53 is a potent transcriptional regulator of cell growth whose induction leads either to cell cycle arrest or apoptosis. Loss of p53 function correlates with cell transformation and oncogenesis. LANA has been found to interact with p53 resulting in a repression of the p53 transcriptional activity. LANA also inhibits the ability of p53 to induce cell death.

It is most probable that LANA may contribute to viral persistence and oncogenesis in KS.

ORF 72/v-cyclin

KSHV v-cyclin is a viral protein that is encoded by ORF 72 and has been found to have sequence similarity to cellular cyclins, in particular the D-type cyclins.

The sequence similarity of v-cyclin to D-type cyclins suggests that the product could also stimulate kinases resulting in their uncontrolled activation leading to deregulation of cellular proliferation and finally to the tumours associated with KSHV diseases. The KSHV cyclin is expressed in BCBL cell lines and also in spindle cells in KS lesions. The ectopic expression of KSHV cyclin in cells was shown to inappropriately activate cyclin dependent kinases CDK-6 and CDK-4 whose activity normally requires cellular cyclins. V-cyclin/CDK-6 complexes strongly phosphorylated pRB at authentic phosphorylation sites including those previously shown to be the hallmark of RB inactivation. It was also shown that v-

cyclin/CDK-6 complex is resistant to CDK inhibitor proteins thus providing a mechanism to further deregulate cell-cycle progression and so providing tumourigenesis potential.
The presence of KSHV-cyclin in Kaposi's sarcoma biopsies indicates that there is a role for the KSHV-cyclin in the tumours and that v-cyclin may possibly have a direct growth-promoting role in these tumours.

ORF 71/v-FLIP

KSHV ORF 71 encodes a v-FLIP (FLICE/FADD-like IL-β-converting enzyme inhibitory proteins) that consist of 2 death domains and have a putative inhibitory effect of Fas/CD95-mediated apoptosis. It was shown that KSHV v-FLIP protects cultured cells from soluble and membrane bound FasL induced apoptosis by inhibiting caspase activation. Expression of v-FLIP in a FasL sensitive parental cell line allowed in vitro clonal cell growth in the presence of FasL death stimuli. In vivo, the HHV-8-vFLIP was shown to act as a tumour progression factor. When immunocompetent mice were injected with a tumourigenic cell line transduced with the v-FLIP encoding gene, tumours developed much faster in comparison to the mock transduced cells lacking v-FLIP gene. The rate of apoptotic tumour cells were lower in experimental tumours with v-FLIP expression than that of the tumours lacking v-FLIP.
Since KSHV ORF 71 mRNA encoding v-FLIP is present in the vast majority of KS spindle cells, this suggests that HHV-8 v-FLIP may contribute to the development and progression of KS.

ORF K12/kaposin

This ORF putatively encodes a small hydrophobic antigen consisting of 60 amino acids. It is translated from an abundantly expressed mRNA in KSHV infected BCBL cell lines and KS spindle cells. It was shown that kaposin gene induced tumourigenic transformation of transfected rat fibroblasts. Rat fibroblast cell lines that were transformed with kaposin produced high grade, highly vascular, undifferentiated sarcomas upon subcutaneous injection of athymic mice. Tumour derived cell lines from the latter sarcomas expressed kaposin mRNA, suggesting that it also has a role in the maintenance of the transformed phenotype.

The oncogenic potential of kaposin under experimental conditions suggests its possible role in the development of KS.

Angiogenesis and HHV-8/KSHV

Interestingly two lytic genes were reported to have angiogenic effects. A minor population of lytically infected cells (2-5% of spindle cells of the KS lesions enter lytic viral replication) are important in sustaining the neoangiogenic feature of the disease or in recruiting new, infected tumour cells into the KS lesion directly or via paracrine mechanisms.

Orf 74/v-GPCR

In KSHV the ORF 74 gene has a high sequence homology to cellular interleukin-8 receptor. Remarkably, and in contrast to other viral or cellular chemokine receptors known at present, KSHV ORF 74 appears to be constitutively activated. In addition it can induce cellular signalling in the absence of known chemokine stimulation (agonist independent signalling). Enhanced cellular proliferation and transformation of rat fibroblasts expressing KSHV v-GPCR has been shown. Stable cell lines expressing v-GPCR were tumourigenic in nude mice and present with vascular tumour phenotype. v-GPCR transgenic mice also developed vascular tumours reminiscent of KS. Cells expressing this chemokine receptor homologue had increased vascular endothelial growth factor secretion.
These findings indicate that v-GPCR may contribute to the vascular phenotype of KS and the development of tumour formation.

vMIPs

KSHV encodes at least three putative proteins (vMIP-I-II-III) with significant protein sequence similarity to the cellular chemokine, macrophage inflammatory protein (MIP) 1α.
Both vMIP-I and vMIP-II were shown to inhibit HIV-1 cellular entry by blocking one of the several HIV co-receptors. KSHV vMIPs may influence HIV pathogenesis in vivo. In a chicken chorioallantoic membrane assay both vMIPs induced angiogenesis, which may suggest a possible role in the neoangiogenesis characteristic of KS lesions. vMIP-II was

reported to be a selective Th2 chemoattractant. Most recent data also reported that v-MIP-III stimulates angiogenesis and selectively chemoattracts Th2 cells. KS lesions are characterised by a prominent Th2 infiltrate.

The expression of vMIPs might represent a useful strategy by which KSHV skews an immune response from Th1 to a Th2 environment to subvert host immunity. Besides modulating leukocyte migration into infected tissues v-MIPs may also contribute to the angiogenesis feature of KS lesions via their paracrine effect.

Transformation of endothelial cells by KSHV

In a recently published study primary human endothelial cells were infected with purified KSHV particles. Infection caused long term proliferation and survival of infected umbilical vein endothelial cultures. The increased lifespan was associated with the acquisition of telomerase activity indicative of indefinite proliferation. Colonies forming in soft agar were also observed indicative of anchorage independent growth. Interestingly only a minor portion (2-5%) of the cells had detectable latent KSHV antigens but the virus negative cells changed from cobblestone to spindle cell-like morphology in the infected culture. This latter phenomenon emphasises the importance of paracrine mechanisms in maintaining the phenotypic change and increased proliferation. The downregulation of vascular endothelial growth factor receptor 2 (VEGFR-2) was inhibited via paracrine mechanism in these cultures. VEGFR-2 downregulation is thought to be important in the senescence and death of endothelial cells.

In a study where human dermal microvascular cells were infected with KSHV the latent infection of cells were linked to spindle cell like transformed phenotype. These KS-like cells also showed characteristics of transformed phenotype, such as loss of contact inhibition and acquisition of anchorage dependent growth.

These results provide an excellent in vitro model for viral KS pathogenesis and prove that KSHV is an oncogenic virus that contributes to the development of KS.

Epidemiology of HHV-8 infection

The detection of the HHV-8 genome by polymerase chain reaction (PCR) in DNA specimens from various tissues and by serology to detect anti-HHV-8 antibodies in different populations

has been used to confirm the link between KS and HHV-8 and also to define the epidemiology of HHV-8 infection.

PCR epidemiology

Using PCR technique, HHV-8 can be detected in up to 100% of KS tissue samples irrespective of its clinical stage or the epidemiological form of the disease as mentioned previously. HHV-8 DNA has been reported in association with a subset of multicentric Castleman's disease and in AIDS-related body-cavity-based lymphomas (BCBL). Both of these lymphoproliferative disorders are rare, but are more prevalent in HIV positive KS patients. BCBL cell cultures have great importance in the serology of HHV-8. On the contrary HHV-8 DNA has not been found frequently in tissue specimens from other diseases or from various specimens from healthy subjects. Most of the PCR results from reliable laboratories indicate that there is an epidemiological link between HHV-8 and KS. The prevalence of HHV-8 by PCR from non-KS specimens such as PBMC, semen or other non-KS tissues is largely determined by the geographical area from which the specimens were obtained from. The virus is more prevalently detectable from healthy persons where KS is prevalent or endemic. It is widely accepted that the epidemiology of HHV-8 infection is more accurately described by seroepidemiological studies as summarized later.

HHV-8/KSHV antigens

LANA

In vitro cultured body cavity lymphoma cells (BCBL) harbour latent HHV-8 and express HHV-8 latent antigens. These cells were the source of KSHV antigens in these first generation assays (IFA, WB). Most KS sera tested by IFA on BCBL cells were found to react with nuclear antigens. The positive KS sera showed dotted/spotted/punctate immunoreactivity on various BCBL cells. These antigen/antigens with the characteristic speckled pattern were referred to as latency associated nuclear antigen (LANA). It was suggested that it is a virally encoded HHV-8 specific antigen since the same set of sera did not reacts with herpesvirus negative cells by the same assay and the patients were readily infected with HHV-8 as shown by the apparent KS and positive PCR results. On Western blot analysis anti-LANA positive

sera reacted with a high molecular weight nuclear antigen of 222-234 kDa from nuclear extracts of BCBL cells. It was revealed that latent ORF 73 transcript encodes this high molecular weight latent nuclear antigen LANA/LNA-1. The 222-234 kDa antigens recognised by KS sera and the speckled nuclear antigen are the same.

sVCA

The high concordance of seropositivity to latent nuclear antigen with the epidemiology of Kaposi's sarcoma made the anti-LANA assay to be the most widespread method in HHV-8 serology, however the correct epidemiology of the virus required assays with additional antigens. Immunofluorescence assays detecting antibodies to lytic viral antigens showed enhanced sensitivity with debated specificity. Small virus capsid antigen (sVCA) vp19 encoded by orf65 was identified as an important immunogenic structural antigen of HHV-8. The major immunogenic epitope was mapped to its carboxyterminus. The orf65 antigen is used in HHV-8 serology because cross reactivity with the corresponding EBV BFRF3 antigen was not detected. Studies with orf65 antigen showed concordant results with anti-latent nuclear antigen serology in Kaposi's sarcoma groups. The orf65 assays showed that the HHV-8 seroprevalence of the healthy population was about the same as the prevalence of antibodies to latent nuclear antigen. However the two assays were less concordant on the level of individual samples in the blood donor than in the Kaposi's sarcoma group.

gp 35-37

The glycoprotein antigen gp35-37 encoded by orfK8.1 was identified as another major lytic antigen of HHV-8. The gp35-37 antigen is a virion envelope glycoprotein. Lytically induced BCBL-1 cultures abundantly express this gp35-37 antigen encoded by the K8.1β spliced transcript. The K8.1 envelope antigen is located in the cellular membrane of lytically induced HHV-8 infected BCBL cells and the immunogenic part of K8.1 resides on the extracellular domain since immunofluorescence assay shows membrane fluorescence on 10-15% of non-permeabilised lytic BCBL cells by anti-K8.1 antibody.

Seroepidemiology of HHV-8

In order to assess the seroprevalence of HHV-8 in the general population and in KS risk groups and to reveal the possible routes of the infection, several serological assays detecting antibodies to different HHV-8 antigens were developed. In this paragraph we describe the results of the first generation seroassays detecting antibodies to HHV-8 latent nuclear antigens (LANA). Anti-LANA serology is the most frequently used assay and its results are widely accepted. Anti-LANA antibodies can be detected by Western blot (WB) or indirect immunofluorescent assay (IFA). These assays have high sensitivity and specificity for detecting KSHV infection. In different KS groups anti-LANA serology showed 80-100% seropositivity of the patients. In follow up studies of HIV+ patients seroconversion to anti-LANA occurred prior to clinical KS development. It was found that HHV-8 also infects the general population and is not solely restricted to KS patients. Prevalence of anti-LANA antibodies is consistent with the geographic distribution of KS. In Central-Africa where KS is endemic anti-LANA seroprevalence was about 50-70%, whereas in Italy where the Mediterranenan classic form is prevalent antibodies were detectable in 20-30% of the normal population. Western-European countries and North America showed 1-5% prevalence of antibodies. There is a decreasing geographical gradient of HHV-8 prevalence from Africa through the Mediterraneans to Europe and North America corresponding to a similar geographical distribution of KS.

The most prominent KS risk group, the HIV+ KS negative male homosexuals, showed an elevated anti-LANA prevalence (approximately 30%) in contrast to the slightly KS affected HIV+ haemophiliacs. In the male homosexual group the number of homosexual partners and intercourses correlate with the seroprevalence rate. The sexual transmission route of HHV-8 in the general population where KS is non-endemic was proven by the elevated seroprevalence of STD clinic attendants compared to the given normal population. Recent data from HHV-8 endemic areas revealed that the virus transmission is not only restricted to sexual practices, but is also linked to other contact transmission routes as in case of other herpesviruses.

The titres of antibodies to LANA also indicate a difference between the seropositive healthy subjects and KS patients. Healthy subjects or patients with non-KS malignancy tend to have a low titre of antibodies contrary to the very high titre of anti-LANA antibodies in KS patients.

There is emerging data in the literature that serologic tests using other antigens of HHV-8 (e.g. ORF 65/small virus capsid antigen and ORF K8.1/envelope glycoprotein antigen) show consistent results with anti-LANA serology and KS epidemiology.

AIMS

- Detection and characterisation of HHV-8 infection of Hungarian classical KS patients.

- Description the epidemiology of HHV-8 infection of Hungarian blood donors by different serological methods.

METHODS

- Detection of HHV-8 DNA by PCR and nested-PCR in classical KS samples,
- SSCP analysis of amplicons,
- Sequencing of amplicons.

- Detection of HHV-8 IgG antibodies by anti-LANA IFA,
- Detection of HHV-8 IgG antibodies by orf65 carboxyterminal peptide ELISA,
- Setting up recombinant orf65 and recombinant K8.1 ELISA,
- Detection of HHV-8 IgG antibodies by recombinant ELISA.

- Statystical analysis by Fischer-, T-, Mann-Whitney-tests and regression analysis.

RESULTS

Detection of HHV-8 in clinical samples

Twenty-five of 28 classic KS samples were positive for HHV-8 by nested PCR (Table I). HHV-8 was not detected in non-KS angiomatous tissues such as in benign angiomas, pyogenic granulomas and in non-angiomatous tumours, namely melanoma and basalioma. HHV-8 was detected in all stages of KS, but at lower frequencies in early stage lesions (Table I). The small number of KS cells in these sections may lead to the lower rate of HHV-8 detection. The high prevalence of HHV-8 in KS versus non-KS tissues and the distribution of HHV-8 in all stages of KS support its possible pathogenic role in KS as suggested by others.
Several genetic variants as point mutations are often within this region as showed by others' results. Sequence analysis of the amplified products were performed in case of multiple (total of 21) samples of 6 patients. SSCP analysis of PCR products revealed 4 distinguishable different SSCP patterns (a, b, c, d) representing different point mutations in the amplified region. Sequencing analysis revealed the sensitivity and specificity of the SSCP analysis. Sequencing of 3 different SSCP patterns (a, b, c) revealed 4 different sequence variants (Table II.). Samples belonging to the same patient regardless of the sampling time or the localisation of multiplex lesions showed uniform SSCP patterns and sequence variants [Table II.]. Due to the uniform SSCP patterns of different KS samples of the same patient, the lesions should contain the same sequence variant of HHV-8.

Table I. Sample stage and HHV-8 detection in classical Kaposi's sarcoma samples by nested PCR.

Stage	HHV-8 +	HHV-8 -	Total
patch	1	2	3
plaque	5	1	6
nodular	19	0	19
Total	25	3	28

Table II. Point mutations in the sequenced region compared to the published sequence

Nucleotide changes	SSCP pattern
1033. C>T 1146. G>A	a.
1032. C>A 1033. C>T 1132. A>G 1139. A>C	b.
1033. C>T	c.
1033. C>T 1145. G>A	a.

Seroepidemiology of HHV-8 in Hungary

The seroprevalence rate was 1.56% (17/1089) in the healthy adult Hungarian population by the immunofluorescence assay. Antibodies to latent nuclear antigen were not demonstrated in 29 children under 14 years. 13 of the 17 blood donors with antibodies to latent nuclear antigen were male (male:1.92%, female: 0.96%), however the difference in distribution between sexes was not significant (Fisher-exact $p>0.2$). The mean age of the seropositive blood donors was 42.7 years and was statistically different from that of the seronegative donors (Mann-Whitney $p=0.045$). To clarify the role of age on anti-latent nuclear antigen seroprevalence, regression analysis was performed. It revealed that the age has moderate but statistically significant effect on seropositivity in blood donors without Kaposi's sarcoma (OR=1.05/year, CI:1.0005-1.1069, $p=0.048$ and see Figure 1.).

In contrast to the normal population, all 12 classical Kaposi's sarcoma patients had antibodies to latent nuclear antigen. The difference between the seroprevalence rates of the blood donors and the Kaposi's sarcoma group was significant (Fisher-exact-test, $p<0.01$). The titers of antibodies to latent nuclear antigen were also different in the two groups (Mann-Whitney $p<0.001$). Most of the seropositive blood donors had lower titers than that of the Kaposi's sarcoma patients (Table III.).

The anti-capsid seroprevalence rate was 0.83% (4/482) in the healthy blood donor group by the orf65 peptide ELISA. Three of them were male and only one was female which represents similar distribution of seroreactivity between the genders as determined by the immunofluorescence assay. Initially, 6 sera had reactivity higher than the cut off value (OD=0.2), 4 of them were positive also on recombinant orf65 antigen Western blots. Only one of the 20 randomly chosen ELISA negative samples (OD<0.2) showed reactivity on the Western blot. In 9 of the 12 classical Kaposi's sarcoma cases, antibodies were detected against the orf65 peptide and recombinant antigen. The different prevalence of anti-capsid antibodies between the blood donor and Kaposi's sarcoma groups were also significant (Fisher exact-test, $p<0.005$). The age distribution of antibodies detected by the peptide-ELISA fits to the age distribution of antibodies to latent nuclear antigen (Figure 1.). The low number of positive results by this ELISA did not permit further statistical analysis in connection with age. The overall HHV-8 seropositivity rate in the blood donor group by the peptide-ELISA and the immunofluorescence assay was 1.86% (9/482).

The agreement of the ELISA and the immunofluorescence assay results in the Kaposi's sarcoma group is much better than in the blood donor group (Table IV.). Our findings are similar to others' results where the orf65 assay similarly gave less concordant results with anti-latent nuclear antigen serology on the level of individual samples from healthy blood donors.

Table III. HHV-8 seroprevalence rates in different groups

	orf65 petide ELISA*	**Antibodies to latent nuclear antigen**	
	prevalence	**prevalence**	**titre (median)**
children	not done	0/29 (**0%**)	-
blood donors	4/482 (**0.89%**)	17/1089 (**1.56%**)	40-1280 (**120**)
KS	9/12 (**75%**)	12/12 (**100%**)	640-10240 (**2560**)

* confirmed by recombinant orf65 antigen Western blot

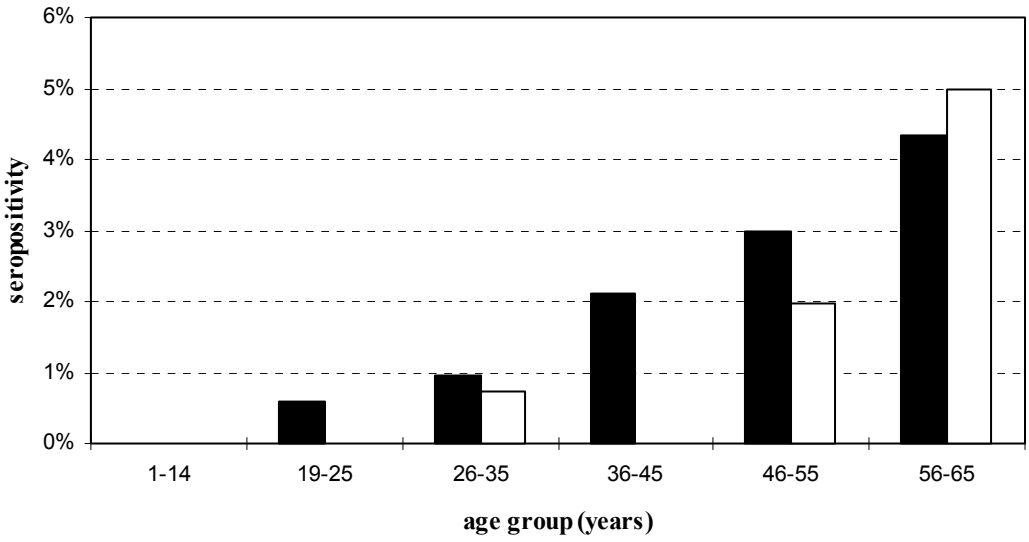
Table IV. Comparison of results by peptide-ELISA and by immunofluorescence assay

		orf65 peptide-ELISA confirmed by recombinant Western blot			
		Kaposi's sarcoma patients (n=12)		**blood donors** (n=482)	
		+ ELISA	**- ELISA**	**+ ELISA**	**- ELISA**
Immuno-fluorescence assay	+	9	3	2	7
	-	0	0	2	471

(Measure of agreement by kappa test between the assays in the blood donor group: κ=0.3)

Figure 1. Distribution of HHV-8 antibodies in age groups.

Anti-capsid antibodies were detected by orf65 peptide ELISA (□), analysis of 472 donors sera n=0, 72, 135, 143, 102, 20 in the different groups, respectively. Antibodies to latent nuclear antigen were detected by immunofluorescence assay (■), analysis of 1089 donors and 29 children's sera. The number of tested samples were 29, 166, 312, 330, 235, 46 in the groups, respectively.



DISCUSSION

On the basis of the clinical behaviour it was unclear whether KS is a true neoplasm or a multicentric reactive hyperplasia. Reactive proliferative origin rather than a clonal neoplasm was supported by flow cytometric DNA analysis by Feulgen-based DNA analysis and by cyclin immunochemistry. Polyclonality was shown by different chromosomal abnormalities of primary AKS cultures. Recently clonality of spindle cells was suggested by the monoclonal pattern of inactivation of X chromosomes and by the uniform methylation pattern of androgen-receptor gene in AKS. As shown above the clonal or polyclonal origin of KS is controversial. The analysis of cellular genomic DNA content of KS lesions may lead to opposite results even with the same method since lesions contain various cell populations. The spindle cells of KS lesions harbour the HHV-8 genome in latent circular form. The consequence of viral latency are the minimal level of replication and infrequent mutations, so HHV-8 DNA analysis would be suitable for judging the clonality of KS. The consistent SSCP patterns correspond well with the latent viral phase and suggest the clonal origin of different lesions from the same patient. In case of polyclonal origin several different sequence variants represented by different SSCP patterns would be detected.
In addition, the peripheral blood mononuclear cells (PBMCs) of KS patients are lytically infected with HHV-8. During the lytic phase in the PBMCs new point mutations occur in the HHV-8 genom compared to that of the lesions. It is not probable that the same sequence variant of HHV-8 from lytically infected PBMCs infects secondary several lesions independent from each other in place or time. In summary, the latent HHV-8 infection of KS lesions is restricted to spindle cells so the consistent SSCP patterns of HHV-8 indicate the clonal origin of different KS lesions. Our results also supports the theory of the pathogenic role of HHV-8 in KS. Neoplastic spindle cell clone could emerge from HHV-8 infected cells eg. endothelial cell cells or its precursors angioblasts.

The overall 1.86% seroprevalence indicates that HHV-8 infection is slightly more prevalent in the Hungarian population than suggested only by latent nuclear antigen serology [Juhász et al., 1998]. This seroprevalence rate fits well into the geographic gradient of HHV-8 seroprevalence which declines from Africa (30-70%) through the Mediterranean (10-30%) to Western-European countries (1-2%). It was shown also in the Italian population that the seroprevalence rates were increasing similarly with elder age. The different distribution of

seroprevalence between the sexes and its increase with age were also described by others. The distribution of HHV-8 seroprevalence in the age groups and between the genders are also in good agreement with the infrequent clinical manifestation of classical Kaposi's sarcoma. Classical Kaposi's sarcoma appears rarely and mainly in elderly male patients. Kaposi's sarcoma patients in this study were significantly older than the seropositive blood donors (Mann-Whitney, $p<0.001$). According to the regression model the hypothetical prevalence of antibodies to latent nuclear antigen in the blood donor group would be 4,8-13% at the age of 60-80 years (the typical age of classical Kaposi's sarcoma patients). Most likely classical Kaposi's sarcoma is a rare consequence of HHV-8 infection after long incubation period as suggested by others.

Orf73 encoded latent nuclear antigen is highly expressed in Kaposi's sarcoma tissue so this could be the explanation of the observed high titer of antibodies in the classical Kaposi's sarcoma patients in contrast to the typical low titers in healthy subjects. The higher titer of antibodies to latent nuclear antigen in patients with Kaposi's sarcoma than in patients with other malignancies or blood donors were also reported in a population with high HHV-8 seroprevalence. The titer of antibodies against latent nuclear antigen may also indicate the clinical progression of Kaposi's sarcoma.

In 2-5% of the cells of a Kaposi's sarcoma lesion lytic viral cycle occurs so the tumour itself provides structural viral antigens. This could be the trigger of the anti-capsid seroresponse, which was demonstrated in 75% (9/12) of the classical Kaposi's sarcoma patients. The lower concordance between the two assays in the healthy blood donor group is probably caused by the different sensitivity of the assays. Several factors such as the large number of immunogenic epitopes on latent nuclear antigen versus the single epitope on the oligopeptide and the unknown time-course of the seroresponse to this antigens could be important in determining sensitivity. A recent study with an almost identical orf65 carboxyterminal oligopeptide also described the inconsistency of the peptide-ELISA in serial samples from 8 of 10 women. It can happen that antibodies to this single peptide epitope are only transiently detectable after primary infections or viral reactivation intervals when increased lytic virus replication is present. The level of antibodies to this single linear epitope may correlate with the virus load of a given patient. These questions require further confirmation. Most likely, the overall rate of HHV-8 infection in the Hungarian population is still underestimated by these two assays. Other important structural antigens such as orfK8.1 envelope glycoprotein

should be also used in order to obtain more accurate prevalence of HHV-8 infection in the general population.

SUMMARY

Human herpesvirus-8 (HHV-8) is the infectious agent of Kaposi's sarcoma irrespective of its clinical or epidemiological form.

HHV-8 was detected in 25 of 28 formalin-fixed paraffin embedded classical Kaposi's sarcoma samples by nested polymerase chain reaction. In case of six patients multiple samples (n=21) were available. Single strand conformational polymorphism (SSCP) of amplicons revealed that samples belonging to a given patient harbour the same sequence variant of HHV-8. SSCP results were confirmed by sequencing in some cases.

To assess the prevalence of HHV-8 infection in the Hungarian population serologic tests were developed and performed with sera of Hungarian blood donors and classical Kaposi's sarcoma patients as positive controls. Sera were tested against all major serological antigens of HHV-8 such as latent nuclear antigen (LANA), orf65 encoded small virus capsid antigen (sVCA) and orfK8.1 encoded virion envelope glycoprotein gp35-37. Indirect immunofluorescence assay were used to detect IgG antibodies to LANA. To detect IgG antibodies to structural antigens we used recombinant recombinant orf65 and orfK8.1 antigen ELISA and orf65 peptide ELISA. ELISA results were confirmed by Western blot.

The reactivity of sera from classical Kaposi's sarcoma patients to LANA, sVCA and K8.1 antigen were 15/16, 11/16 and 14/16, respectively. In the blood donor group we detected antibodies to LANA, sVCA and K8.1 antigen in 17/1089, 7/482 and 6/482 cases, respectively. The overall seroprevalence of HHV-8 of Hungarian blood donors (n=482) and classical Kaposi's sarcoma patients (n=16) was 3.52% and 100%, respectively. The prevalence of HHV-8 specific antibodies showed a moderate but significant increase with senescence.

Our results correspond well with the role of HHV-8 in Kaposi's sarcoma and the 3.52% HHV-8 seroprevalence rate of Hungarian blood donors fits to the decreasing rate of HHV-8 seroprevalence from the HHV-8 endemic African countries thorough the Mediterranean to Western Europe.

Thesis based on the following publications:

A Juhasz, E Remenyik, K Szarka, G Veress, J Hunyadi and L Gergely: *Consistent PCR-SSCP pattern of HHV-8 in the course of classical Kaposi's sarcoma assumes its clonal origin*, J Med Virol, 54:300-304, 1998

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