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Studies on Human Polyomavirus Infection in Immunosuppressed Patients and in Patients with Polyoma Related Tumors

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Stockholm 2001

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-031-8

Abstract

Polyomaviruses are potentially oncogenic viruses found in humans, in other mammals and in birds all over the world. The polyomaviruses that have been observed in humans are BK virus (BKV) and JC virus (JCV) as well as the primate polyomavirus Simian Virus 40 (SV40). BKV and JCV persistent latent in humans, but are not believed do not cause any disease or symptoms in immunocompetent individuals. However, in immunosuppressed individuals, e.g in bone marrow transplanted (BMT) patients BKV has been reported to be associated with hemorrhagic cystitis (HC), and in HIV positive patients JCV has been shown to cause progressive multifocal leukoencephalopathy (PML). SV40 is believed to have been transferred to humans via contaminated polio vaccine 1955-63, and recent studies have detected fragments of SV40 DNA in malignant mesotheliomas (MM), brain tumors and osteosarcomas. However, the reported frequency varies widely between different studies and countries. The aim of the present study was to further investigate human polyomavirus infections in immunosuppressed patients and in patients with polyoma virus related tumors.

In BMT patients with late onset HC, BKV is excreted in the urine, but all BMT patients with BK viruria do not develop HC. To investigate if primary BKV infection transmitted from donor to recipient during BMT could cause HC we investigated anti BKV serum titers in BMT recipients and their donors. We also sequenced the non coding control region (NCCR) and the VP1 region of BKV from BMT patients to search for mutations correlated to the development of HC. Primary BKV infection was not the major cause of HC in BMT patients since all patients had anti BKV antibody titers before BMT. However, after BMT significantly more patients with HC had serological changes compared to patients without HC. BKV with C to G mutations in the NCCR Sp1 binding site was significantly over represented in BMT patients with HC. However, when we by Real Time PCR investigated if these mutations resulted in a higher BKV load in the urine of affected patients, we found that this was not the case.

To study if human polyomavirus reactivation was related to graft cold ischemia time and if this could cause graft rejections in renal transplant (RTX) patients, we analyzed urine samples by PCR from RTX patients for presence of BKV or JCV and in parallel we examined their files for graft rejection. Reactivation of BKV or JCV was not correlated to the length of the cold ischemia time or to complications after RTX, such as rejection.

JCV can be detected by PCR in the cerebrospinal fluid (CSF) of patients with PML. To investigate if JCV is present in CSF during other central nervous system (CNS) infections or in Multiple Sclerosis (MS) patients, we analyzed CSF samples by PCR from patients with other viral CNS infections and MS. JCV was not detected by PCR in CSF from patients with HSV-1 encephalitis, enteroviral meningitis, nonenteroviral meningitis or during MS. Thus, detection of JCV by PCR in CSF is indicative for PML.

In order to investigate if SV40 or human polyomaviruses were present in Swedish malignant mesotheliomas (MM), and possibly related to the distribution of early batches of SV40 contaminated polio vaccine, we analyzed Swedish MM samples for SV40 by PCR. SV40 was present in only 10% of the Swedish MM (from three men born 1909-1929), which was less than that reported, 40-69% in Italy and the USA. This indicates that SV40 contaminated polio vaccine not was distributed widely in Sweden even before 1958.

ISBN 91-7349-031-8

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VI. Presence of Simian Virus 40 (SV40) is not a frequent finding in Swedish malignant mesotheliomas. Priftakis P, Bogdanovic G, Hjerpe A, Dalianis T. *Submitted to J. Clinical Microbiology.*

Abbreviations

Ab	antibody
BKV	BK virus
BMT	Bone marrow transplantation
bp	base pair
CD	Cadaver donor
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computor tomography
ELISA	Enzyme linked immunosorbent assay
GVHD	Graft versus host disease
HAART	Highly active anti retroviral therapy
HAI	Hemagglutination inhibition
HIV	Human immunodeficiency virus
HLA	Human leukocyte associated antigen
HC	Hemorrhagic cystitis
IgG	Immunoglobuline G
IgM	Immunoglobuline M
IVIG	Intravenous immunoglobuline infusion
JCV	JC virus
LD	Living donor
LT	Large T-antigen
MM	Malignant mesothelioma
MRI	Magnetic resonance imaging
NCCR	Non coding control region
PCR	Polymerase chain reaction
PML	Progressive multifocal leucoencephalopathy
RDE	Receptor destroying enzyme
RTX	Renal transplantation
ST	Small T-antigen
SV40	Simian virus 40
VP 1 - 3	Viral capsid proteins

1. Introduction

Polyomaviruses are small DNA viruses that are widely distributed in mammals and birds all over the world, for review see Cole 1996. The viruses are very well adapted to their hosts, and most probably polyomaviruses and their hosts have co-evolved for a long time during evolution. In humans, natural infection occurs with JC virus (JCV) and BK virus (BKV). JCV was isolated in 1971 in a patient with progressive multifocal leukoencephalopathy (PML) (Padgett et al. 1971). Later the same year BKV was isolated from a renal transplant patient (Gardner et al. 1971). The designation of these viruses comes from the initials of patients from where they were isolated. The primate polyomavirus, Simian Virus 40 (SV40), has been detected in human tumors, and is believed to have been transferred to humans via contaminated polio vaccine or by close contact with rhesus monkey. Primary infection and persistence of BKV or JCV are asymptomatic in healthy individuals. However in immunosuppressed individuals, infection or reactivation of these viruses can cause severe diseases and conditions for review see Cole 1996 and Shah 1996. JCV can induce PML in immunosuppressed patients. PML was a rare disease occurring only in some patients with lymphoma, prior to the AIDS epidemic; however since 3-8% of all HIV positive patients present PML the incidence of PML has increased dramatically. BKV is associated to hemorrhagic cystitis (HC), a condition occurring in some patients after bone marrow transplantation (BMT). It has also been suggested that reactivation of BK and JCV could induce complications in renal transplanted (RTX) patients. Recently, BKV DNA fragments have been found in some human solid tumors, and JCV is under investigation for its role in leukemia, for review see Jasani et al. 2001 and Smith 1997. DNA fragments of SV40 have been found in malignant mesothelioma (MM) and other human tumors. However, others have challenged the clinical relevance of the connection between SV40 infection and tumor development, for review see Jasani et al. 2001.

The general aim of this study was to further investigate BKV and JCV infections in immunsuppressed patients, more specifically in BMT patients, RTX patients and patients with PML. Furthermore, we also aimed to investigate the role of SV40 infection in Swedish patients with MM.

2. Specific aims

2.1 Studies on human polyomavirus infection in immunosuppressed patients

► In BMT patients, we wished to further investigate the role of BKV in patients developing HC and we had the following three aims:

- To study if primary BKV infection, possibly transmitted from donor to recipient during allogenic BMT in children with leukaemia, was associated with the occurrence of HC.
- To examine if different BKV strains or mutations, were responsible for the development of HC.
- To investigate if the observed mutations in the NCCR Sp1 binding site BKV from patients with HC were correlated to a higher viral load in the urine.

► In RTX patients, our aim was to investigate if reactivation of BKV/JCV was influenced by the graft cold ischemia time, and if viral reactivation resulted in a higher frequency of graft rejection episodes.

► In patients with PML our aim was to study if presence of JCV DNA in the CSF was diagnostic for PML. More specifically we wished to exclude that JCV was reactivated and excreted in CSF during other viral infections in the CNS or during MS.

2.2 Studies on polyomavirus related tumors

► Our aim was to examine if SV40 or human polyomaviruses were present in malignant mesothelioma in Swedish patients, and to investigate if there was a correlation to the administration of SV40 contaminated polio vaccine in these patients.

3. Human Polyomaviruses and SV40

3.1 General

Polyomaviruses are a subfamily of the Papovavirus family (Fig.1), for a review see Cole 1996. All papovaviruses are small, nonenveloped viruses with an icosahedral capsid. The name *papova* originates from the included virus groups; *pa* for rabbit *pa*pillomavirus, *po* for mouse *po*lyoma and *va* for Simian Virus 40 (SV40), originally called *va*coulating virus (Shah 1990).

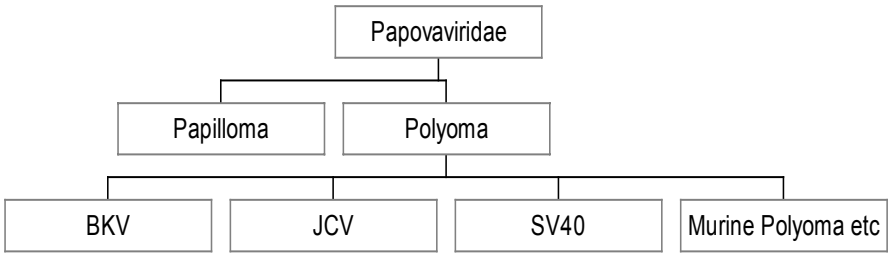

Fig 1. Papovaviridae

All polyomaviruses are similar in genomic size and organization (Pipas 1992). Their DNA is superhelical, double stranded and circular; and their replication occurs in the nucleus. Within the polyomavirus subfamily, murine polyomavirus and SV40 are the most studied members. These two viruses have been used for *in vivo* and *in vitro* studies on the mechanism for cellular transformation, oncogenesis, cell cycle control, viral transcription and replication as well as viral persistence and tumor immunology, for reviews see (Griffin et al. 1983; Dalianis 1990; Berke 1997). In humans natural infection occurs with JCV and BKV. JCV was detected in 1971 in a patient with PML and Hodgkin lymphoma (Padgett et al. 1971), and BKV was isolated from a renal transplant patient (Gardner et al. 1971). The primate polyomavirus, SV40, was originally detected in humans living close the natural host of SV40; the rhesus monkey. However, in 1955-1963 SV40 was accidentally transferred to an unknown number of people via SV40 contaminated polio vaccines. Recently, SV40 DNA fragments have been found in different human tumors, for review see Jasani et al. 2001.

3.2 Virion Structure

Polyomaviruses have a diameter of 40-45 nm and a molecular weight of $3.2x10^6$ Daltons (Shah 1990). The polyomavirus genome is surrounded by an icosahedral capsid consisting of the three capsid proteins VP1, VP2 and VP3 (Fig.2).

Fig 2. The 72 pentameric capsomeres of the polyoma capsid (Frisque 1999).

The major capsid protein, VP1, dominates the capsid by forming 72 pentameric capsomeres, i.e. totally 360 molecules per capsid. (Rayment et al. 1982; Liddington et al. 1991). The VP1 molecules are connected via the C-terminal end of their molecules, and it is known that VP1 has the ability to self-assembly into virus like empty capsids (Salunke et al. 1986). However, in complete virions VP2 and VP3 form complexes with VP1 at their C-terminal ends, while their N-terminal ends extend out from the capsid (Barouch et al. 1994). Nevertheless, only about 30-60 molecules of VP2 and VP3 are present in the capsid (Rayment et al. 1982; Liddington et al. 1991). The result of polyomavirus production leads most commonly to a viral DNA containing virion, where the encapsulated DNA of polyoma forms a chromatin or a "mini-chromosome" in complex with cellular histones H2A, H2B, H3 and H4 (Cole 1996). However, a large proportion of the virions contain defect viral DNA with deletions, duplications, rearrangements or cellular DNA. Furthermore, empty virus capsids are also produced (Cole 1996).

3.3 Viral genome

The polyomavirus genome is divided into an early region, a late region and a NCCR also designated as the Regulatory Region (RR) (Fig. 3 and 4), for review see Cole 1996. The division into early and late regions corresponds to the time after infection when their genes are transcribed. The encoding region of the virus genome is highly conservative, while the NCCR is variable (Seif et al. 1979; Sugimoto et al. 1990). The NCCR is situated between the two encoding early and late regions, and contains the origins of transcription for both the early and late regions. Transcription starts at the sites of initiation and continues bi-directionally to about 180° in the circular DNA (Cole 1996).

The early region encodes the proteins expressed during the early stage of infection, before viral replication. These proteins are large T-antigen (LT) and small T-antigen (ST) in BKV, JCV and SV40. In murine polyoma a middle T-antigen is also present. The late region encodes the viral proteins necessary to form the capsid, VP1, VP2 and VP3, as well as the agnoprotein (LP 1). The genomes of BKV, JCV and SV40 shows high nucleotide sequence homology; BKV and JCV shares 75%, JCV and SV40 as well as BKV and SV40 shares 69% of their sequences (Yang et al. 1979; Frisque et al. 1984).

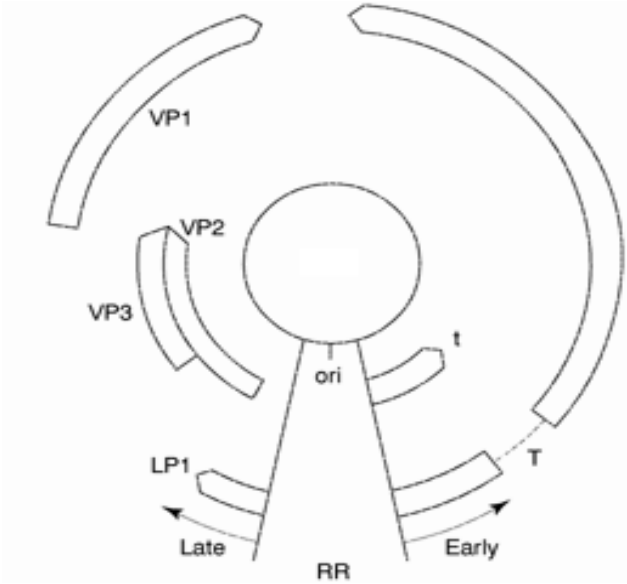

Fig 3. BKV and JCV genom (Frisque 1999)

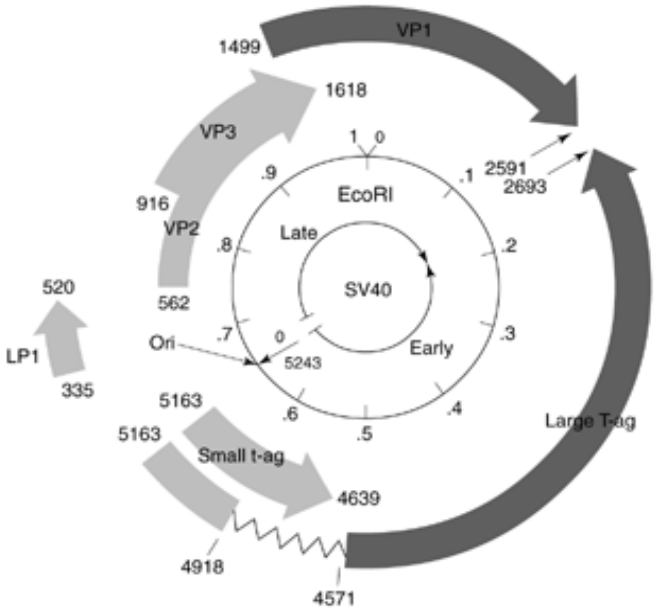

Fig 4. SV40 genome

(Encyclopaedia of Virol. http://apresslp.gvpi.net/apvirol/)

3.3.1 Non coding control region

The NCCR of BKV, JCV and SV40 includes the origin of replication as well as the promoter and enhancer regions including binding sites for cellular transcription factors and LT. The NCCR is highly variable, most likely due to genetic rearrangements as an adaption to different viral environments (Seif et al. 1979; Sugimoto et al. 1990).

In BKV, the NCCR is divided into blocks: the promotor/enhancer blocks O (142 bp), P (68 bp), Q (39 bp), R (63 bp) and a late leader region S (the O and S blocks are not present in Fig. 5) (Ferguson et al. 1994)(Fig.5).

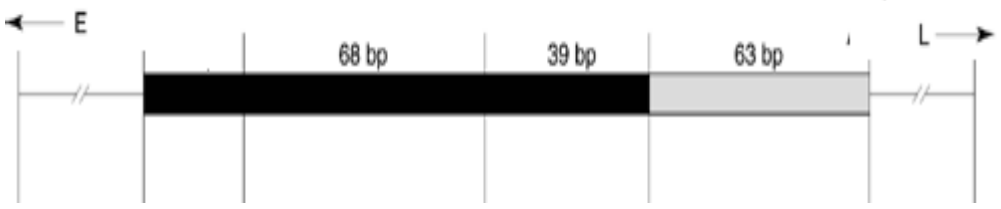

Fig 5. BKV NCCR (Frisque 1999)

Different consensus binding motifs for cellular transcription factors are present in NCCR, e.g. one CRE site in the P block, one Sp1 site in the overlap between the Q and R blocks and three NF1 sites in the P, Q, R and S blocks (Johnsen et al. 1995). Several studies have shown mutations, deletions and duplications in the binding regions for these transcription factors affecting promotor/enhancer function and influencing replication as well as transformation *in vitro* (Yoshiike et al. 1986; Johnsen et al. 1995). The NCCR varies between different BKV strains. These variations have been observed after passage of the virus in cell culture as well as in BKV isolated directly from humans, however it has been difficult to show the clinical relevance of these mutations and rearrangements (Degener et al. 1999).

In JCV extracted from healthy individuals an "archetypal" NCCR organization with six domains: A (25 bp), B (23 bp), C (55 bp), D (66 bp), E (18 bp) and F (the latter is not present in Fig.6) has been observed (Tominaga et al. 1992) (Fig.6).

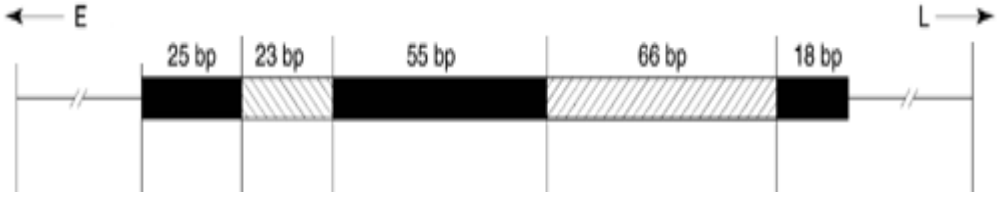

Fig 6. JCV NCCR (Frisque 1999)

Different consensus binding motifs for cellular transcription factors are present in NCCR of JCV, e.g. Tst-1, Sp1, NF1, CRE, TAR, p53 and AP1 (Ault 1997). Immunosuppressed individuals, such as BMT or RTX patients have a higher frequency of the rearrangements in the NCCR as compared to healthy individuals (Myers et al. 1989; Kitamura et al. 1994). In PML patients the JCV NCCR shows a duplication of the A-domain and a deletion of the B-domain. It is believed that the rearrangements in PML patients are derived from the archetypal JCV (Ault 1997).

The SV40 NCCR consists of a 72 bp enhancer (duplicated in laboratory strains), a domain with GC rich 21 repeats and an ORI-region (Fig.7) (Stewart et al. 1998). Also in SV40 several transcription factors bind to this region e.g. Sp1 (Cole 1996). The NCCR of SV40 has been studied extensively, and will be discussed below.

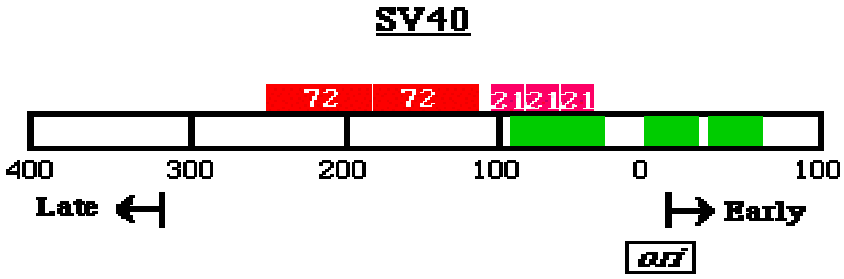

Fig 7. SV40 NCCR (Frisque 1999)

3.3.2 The early region and proteins

The early regions of BKV, JCV and SV40 encode two T-antigens and their designation is based on their size, consequently large T (LT, ~ 688 aa) is larger than small T (ST, ~ 172 aa). The mRNAs of LT and ST are alternatively spliced from a common pre-mRNA. This results in that LT and ST share N-terminal amino acid sequences, but their C-terminal regions are unique.

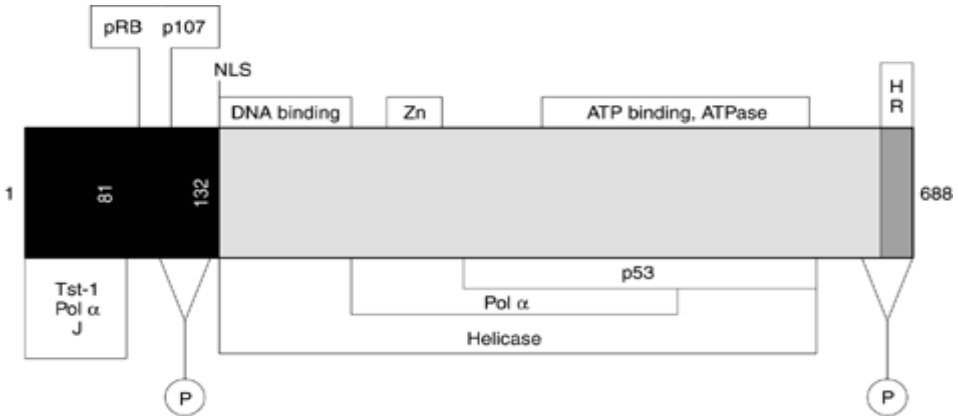

Fig 8. The functions of JCV Large T antigen. (Frisque 1999)

LT is a nonstructural multifunctional protein, which is necessary for stimulation of cellular DNA synthesis and also responsible for the establishment and maintenance of cellular transformation (Shah 1996)(Fig.8). LT binds to three specific LT binding sites in the NCCR to modulate both early and late transcription. Binding at site I regulates early transcription (Rio et al. 1980; Hansen et al. 1981; DiMaio et al. 1982), and binding at site II regulates viral replication, while the function of binding of the low affinity site III is still unclear (Rio et al. 1980; Deb et al. 1987). LT has also the ability to bind several cellular proteins such as DNA polymerase α (Dornreiter et al. 1992), p53 (Lane et al. 1979) and proteins of the Rb-family (DeCaprio et al. 1988), which allows LT to push the host cell into S-phase. Furthermore, LT has a helicas activity (Stahl et al. 1986; Dean et al. 1987) and an ATPas activity (Tjian et al. 1979), which is crucial for replication of the virus. JCV LT shares 83% amino acid homology with BKV and 72% with SV40 (Frisque et al. 1984). Although LT differs between these three viruses, they still cross react antigenically (Takemoto et al. 1973). The function of ST is less known, but it is believed to facilitate the function of LT (Cole 1996).

BKV, JCV and SV40 are predicted to encode totally 7 –10 proteins, and additional small open reading frames have been detected. But so far only an early leader protein (SELP) in SV40, as well as three additional T^1 proteins in JCV, all with unknown function, have been reported (Frisque 1999).

3.3.3 The late region and proteins

The late region encodes three viral capsid proteins VP1 (~ 354aa), VP2 (~344aa) and VP3 (~ 225aa), as well as the agnoprotein (~ 71aa) (Shah 1996). Similar to the early proteins, their mRNAs are derived from a common pre-mRNA by alternative splicing and the coding regions of VP2 and VP3 overlap. The VP1 N-terminal region overlaps with the C-terminal region of VP2 and VP3. However, the reading frame of VP1 is different from that of VP2 and VP3. VP1 is the major structure protein and represents 75% of the viral mass (Shah 1996). It has the ability to attach to cellular receptors, which has shown to be crucial for absorption to the cell membrane. Furthermore, VP1 has the ability to agglutinate human erythrocytes type 0 (Bolen et al. 1981; Shah

1996). As mentioned above the encoding region is highly conservative. Nonetheless, in BKV, minimal variations are seen in the late region encoding the major capsid protein VP1. This variability makes it possible to divide BKV into four different subtypes denoted I - IV (Jin 1993). The function of VP2 and VP3 are less known. However, they are believed to be important for viral adsorption and uncoating (Cole 1996). The agnoprotein, derived from late mRNA, seems to facilitate the transport of VP1 to the nucleus, and may enhance the ability of the virus to spread (Carswell et al. 1986). JCV VP1 has a 78% homology with BKV VP1, and 75% homology with SV40 VP1 (Frisque 1999).

3.4 Viral entry and replication

Viral entry starts with attachment of the virus to the cell surface (Fig.9). The mechanism of attachment is not fully understood. However, for human polyomaviruses, VP1 and sialic acid receptors play a major role (Shah 1996), while for SV40 the major receptor appears to be an MHC class I molecule (Breau et al. 1992). Polyomavirus mainly enter the cytoplasm by pinocytosis, which is preceded by VP2 and VP3 interaction with the cell surface (Cole 1996). The viruses are then transported through the cytoplasm, and enter the nucleus either via the endoplasmatic reticulum (ER), nuclear pores or membrane fusion. In the nucleus the virus is uncoated, a process where VP2 and VP3 also are involved (Cole 1996). Thereafter, the early region on the viral "mini chromosome" is transcribed and the early proteins LT and ST are produced. The proteins drive the cell into S-phase, which is necessary for viral replication, since the viruses need to utilize the cellular replication machinery for replication. LT also binds to the NCCR, which leads to down regulation of early gene transcription and initiation of late transcription as well as viral replication.

As late transcription starts, two sets of mRNA:s are produced, referred to as 16S and 19S (Cole 1996). These mRNA:s are transported to the cytoplasm, where 16S is translated to VP1 and 19S to VP2, VP3 and to some extent also the agnoprotein. The proteins are then transported to the nucleoplasm for assembly, together with the histone associated viral DNA. However, the assembly will also take place without presence of viral DNA. The release of

virions occurs either at cell death or by transport to the surface in cytoplasmatic vesicles (Cole 1996). The cell will increase its replicatory capacity several hundred times in order to process the about 200,000 viral DNA molecules in the infected cell and about half of them will give rise to progeny virions (Cole 1996).

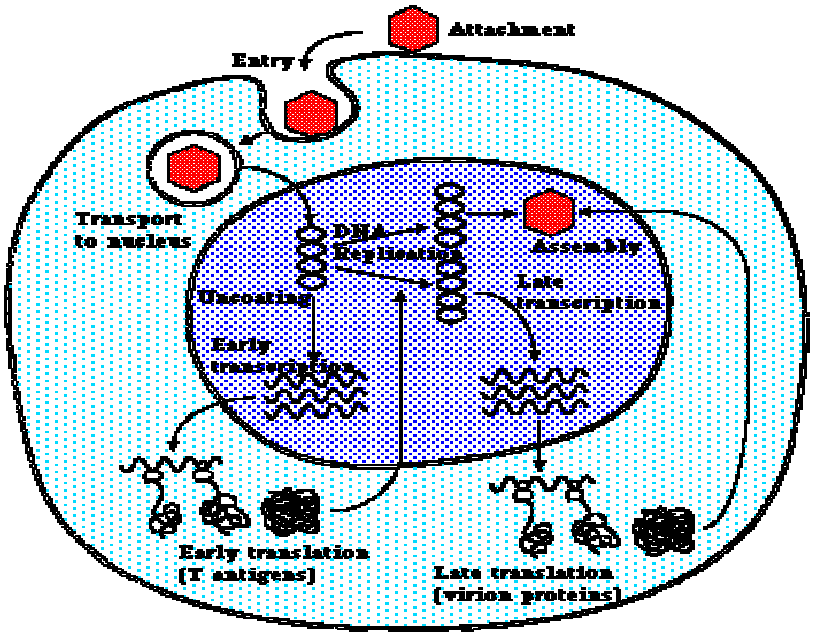

Fig 9. Polyomavirus replication (Frisque 1999)

3.5 Polyomavirus infection

3.5.1 Primary infection

Primary polyomavirus infection is suggested to be symptom free, or to cause mild respiratory symptoms (Mantyjarvi et al. 1973; Goudsmit et al. 1982; Sundsfjord et al. 1994). It has been difficult to isolate the viruses from respiratory tissue, but JCV has recently been found in tonsilar tissue (Monaco et al. 1998; Wei et al. 2000). Epidemiological studies indicate that primary BKV and JCV infections occur in childhood. Transmission of BKV and JCV occurs via humans only, and there is no evidence for an animal reservoir (Padgett et al. 1976).

3.5.2 Persistent infection and reactivation

In immunocompetent individuals, polyomavirus persists in kidneys, peripheral blood and brain (Chesters et al. 1983; Elsner et al. 1992; Tominaga et al. 1992; Tornatore et al. 1992; Dorries et al. 1994). Reactivation occurs more prominently during T-cell deficiency/immunosuppression states, and is followed by excretion of the viruses in urine. Reactivation of BKV during BMT may contribute to HC, and reactivation of JCV in the brain in HIV positive patients during AIDS may cause PML. Reactivation of BKV and JCV during RTX, is suggested to cause post transplantatory complications. Reactivation of JCV and BKV has also been observed in primary immunosuppression disorders and in patients with diabetes (Dörries 1996).

3.5.3 Infection in the kidneys

BKV and JCV are focally distributed within the kidneys (Chesters et al. 1983; Heritage et al. 1981; Tominaga et al. 1992). JCV has been detected in urinary epithelial cells (Randhawa et al. 2001) and BKV and JCV can be propagated in urinary tract epithelial cells (Beckmann et al. 1983). Nevertheless, the cell type in which BKV and JCV persist in the kidneys is still unknown (Shinohara et al. 1993). BKV and JCV DNA are generally episomal and not integrated in the cellular chromosomes (Chesters et al. 1983; Heritage et al. 1981).

3.5.4 Polyomaviruria

Asymptomatic human polyoma viruria is common during primary infection as well as during reactivation. Symptom free excretion of JCV and more rarely BKV does occur in healthy individuals and more prominently at old age and during pregnancy. (Markowitz et al. 1991; Markowitz et al. 1993; Kitamura et al. 1994; Sundsfjord et al. 1994). The reported incidence of human polyoma viruria in pregnant women was 7% for JCV and 15-47% for BKV (Markowitz et al. 1991; Jin et al. 1995). In immunoincompetent patients, such as cancer-, BMT- and HIV positive patients, BK viruria has been reported to vary between 20 - 90%; and JC viruria has been reported to vary between 16 - 67% (Arthur et al. 1986; Cotterill et al. 1992; Markowitz et al. 1993; Azzi et al. 1994; Chan et al. 1994; Sundsfjord et al. 1994; Bedi et al. 1995; Azzi et al. 1996; Bogdanovic et al. 1996; Knowles et al. 1999; Wang et al. 2000). In RTX patients the reported incidence of JC and BK viruria vary between 14 to 65% (Coleman et al. 1973; Lecatsas et al. 1973; Gardner et al. 1984; Marshall et al. 1991; Shah 1996; Priftakis et al. 2000). In summary, JCV can be excreted in urine intermittently in healthy individuals, but the incidence increases in immunosuppressed patients. BKV is excreted in urine generally only during immunosuppression.

3.5.5 Infection in CNS

JCV DNA has been detected in brain tissue of patients with PML, most commonly AIDS patients (Dorries 1984; Elsner et al. 1992; Vago et al. 1996). In patients with PML, JCV could be detected in the CSF. (Hammarin et al. 1996; Vago et al. 1996). However, JCV DNA and VP1 capsid protein have been detected in 20-68% in the brain, but not in the CSF, of patients without PML, as well as in elderly people (Mori et al. 1991; Elsner et al. 1992; Quinlivan et al. 1992; White et al. 1992; Ferrante et al. 1995; Vago et al. 1996). These reports confirm that JCV causes PML and that JCV could be latent in the brain. However, other reports do not support latency of JCV in the brain, while they cannot detect JCV in the brain of non-PML patients (Wiley et al. 1988; Telenti et al. 1990; Henson et al. 1991; Mehal et al. 1993; Perrons et al. 1996).

BKV DNA has been detected in the CNS in a very low frequency by PCR in AIDS patients with and without PML, as well as in nonimmunocompromised patients without PML (Elsner et al. 1992; Ferrante et al. 1995; Vago et al. 1996). Also here contradictory findings have been reported (White et al. 1992). Still no specific disease is connected to BKV infection in the brain.

Finally, SV40 has also been detected by PCR in CSF from PML patients (Tognon et al. 2001) and in patients with different types of brain tumors (Stewart et al. 1998; Huang et al. 1999).

In summary it is still controversial if BKV and JCV persist in the brain and reactivate during immunosupression, or if they are transported to the brain during immunosppression (Bogdanovic 1998). Fewer studies have been made on SV40, and presence of SV40 has only been reported in malignant brain tissue.

3.5.6 Infection in peripheral blood cells and bone marrow

Polyomaviruses were first detected in lymphocytes by electron microscopy of lymphocyte cultures from two patients with measles (Lecatsas et al. 1976). Later JCV DNA has been detected in lymphocytes from HIV infected patients both with and without PML, as well as from HIV negative immunocompromised patients (Tornatore et al. 1992; Ferrante et al. 1995; Dubois et al. 1996; Dubois et al. 1997; Lafon et al. 1998). JCV DNA has also been detected in mononuclear cells (Schneider et al. 1993). Primary receptors for JCV have been found on B-lymphocytes but not on T-lymphocytes (Wei et al. 2000). BKV DNA has been demonstrated in peripheral blood mononuclear cells in AIDS patients both with and without PML (Sundsfjord et al. 1994). Furthermore both BKV and JCV have been detected in peripheral blood cells of immunocompetent individuals (Dorries et al. 1994; De Mattei et al. 1995; Dubois et al. 1997; Dolei et al. 2000), but this has not been confirmed by others (Tornatore et al. 1992; Bogdanovic et al. 1996; Schneider et al. 1993).

In summary, the overall results indicate that BKV and JCV are present in the blood cells of immunocompromised individuals. However it is still unclear if the BKV and JCV persist in the blood cells of non-immunocompromised patients.

3.6 Epidemiology

BKV and JCV are widely distributed in the world, and also represented by different subtypes (Bofill-Mas et al. 2000; Agostini et al. 2001; Jin et al. 1995). The distribution of SV40 is a bit more complex to map, since this virus is believed to have been randomly transferred to humans via contaminated polio vaccine (Bofill-Mas et al. 2000; Carbone et al. 1999). Serological studies have shown that over 50% of all children have antibodies (Ab) against BKV at the age of 3, and against JCV at the age of 10-14 (Flaegstad et al. 1986; Padgett et al. 1973; Shah et al. 1973; Takemoto et al. 1973). The seroprevalence against BKV and JCV is about 60-90% in teenagers (Walker et al. 1983). Thereafter the seroprevalence of BKV declines slowly, while the seroprevalence for JCV increases slowly, to reach around 70-80% for both viruses in adults (Walker et al. 1983; Padgett et al. 1973). (Fig.10)

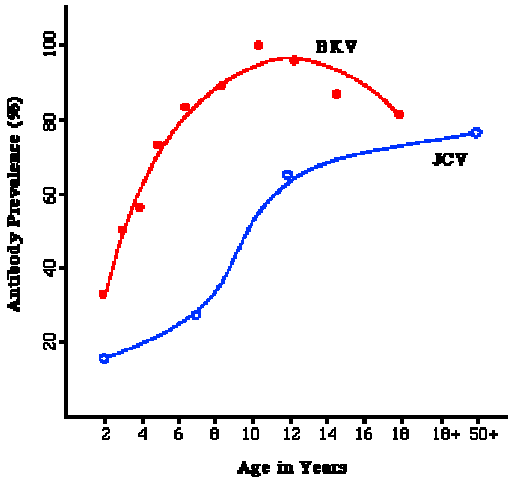

Fig. 10 Age dependent prevalence of antibodies against BKV and JCV (Frisque 1999)

3.7 Human polyomavirus and SV40 related diseases

3.7.1 Hemmorrhagic cystitis in BMT patients

HC is a complication after BMT, with an incidence of 5-34% (Arthur et al. 1986; Ilhan et al. 1997; Childs et al. 1998; Seber et al. 1999; Vogeli et al. 1999). HC is characterized by lower abdominal pain, dysuria, and frequent micturation with presence of hematuria (Arthur et al. 1986). Risk factors for development of HC after BMT are graft versus host disease (GVHD) grade 2-4, use of Busulfan as well as BMT between 10-30 years of age (Seber et al. 1999). Late onset HC, occurring ≥ 2 weeks after BMT, is related to BKV infection or reactivation, while early HC occurring <2 weeks after BMT, is drug associated (Arthur et al. 1986; Apperley et al. 1987; Azzi et al. 1994). Other viruses such as Cytomegalovirus (CMV) and Adenovirus, have also been discussed but have less frequently been associated to late onset HC (Ambinder et al. 1986; Ost et al. 1987; Spach et al. 1993; Akiyama et al. 2001). Both allogenic and autologous BMT patients excrete BKV and JCV in the urine. (O'Reilly et al. 1981; Arthur et al. 1986; Cottler-Fox et al. 1989; Cotterill et al. 1992; Azzi et al. 1994; Chan et al. 1994; Bogdanovic et al. 1996; Azzi et al. 1999). BMT polyomaviruria is dominated by BKV, present in 77-90% of all adult BMT patients both with and without HC (Azzi et al. 1996; Bogdanovic et al. 1996). Thus BKV reactivation alone is obviously not sufficient to cause HC, and different co-factors have been discussed, such as GVHD, primary BK infection and specific BK-strains (Ost et al. 1987; Jin et al. 1995; Bogdanovic et al. 1996; Bogdanovic et al. 1998; Akiyama et al. 2001). Recent studies have shown a higher viral load in the urine of HC patients, indicating a higher replication of BKV, compared to viral load in asymptomatic BMT-patients (Azzi et al. 1999). HC is most often self-limiting and is treated symptomatically with bladder irrigation, analgesia and if necessary with suprapubic catheters and blood transfusions. There are few reports on successful HC treatment with anti-BKV drugs as adenine arabinoside (vidarabine) and cidofovir (Chapman et al. 1991; Held et al. 2000; Vianelli et al. 2000). Also installation of prostaglandin E2 in the bladder has been reported to have effect on HC (Trigg et al. 1990).

3.7.2 Renal transplantation and human polyoma virus infection

BK and JC viruria in RTX patients has been reported to range between 14-65%, and viral reactivation has been suspected to be of either donor or recipient origin (Coleman et al. 1973; Lecatsas et al. 1973; Hogan et al. 1980; Gardner et al. 1984; Marshall et al. 1991; Shah 1996; Randhawa et al. 2001). Post transplantory complications such as ureteral stenosis, graft rejection, rise in serum creatinine level, impaired renal function and PML have been described in RTX patients with polyomaviruria (Coleman et al. 1978; Chesters et al. 1983; Arthur et al. 1989; Shah 1996). However, it has been difficult to assess the clinical relevance of viral reactivation.

3.7.3 Progressive Multifocal Leucoencephalopathy

PML is a subacute demyelinating disease affecting the white matter in the CNS, and it occurs on the background of impaired cellular immunity eg. in HIV positive patients, but also in patients with Hodgkin's disease and tuberculosis (Shah 1996). PML is characterized by mental deterioration, impairment of speech and vision and motoric weakness (Adams 1997). The disease progresses rapidly with paralysis of the limbs, sensory dysfunction, cortical blindness, dementia, coma and death (Berger et al. 1987). JCV is the causative agent of PML, causing a cytocidal infection in the oligodendrocyes (Shah 1996). PML diagnosis is based on clinical findings, histological findings in brain biopsy, Computed Tomography (CT) or Magnetic Resonance Imaging (MRI), and more recently by detection of JCV DNA in CSF by PCR. Today in Sweden, brain biopsy is rarely or never performed to confirm PML diagnosis.

JCV DNA isolated from PML patients show rearrangements in the NCCR, such as a duplication of the A domain and a deletion of the B domain (Shah 1996; Pfister et al. 2001).

It is not clear how JCV enters the brain, but it is suggested that JCV reaches the brain via hematogenous spread from the kidneys after reactivation (Tornatore et al. 1992). This is supported by the fact that the characteristic damages in the grey-white matter junction, is a place of intensive circulation (Major et al. 1995). Another possibility is that JCV persists in the brain and becomes reactivated during immunosuppression (Elsner et al. 1992; White et al. 1992). The demyelinisation occurs focally in the white matter near the grey-

white conjunction (Whiteman et al. 1993). The affected oligodenrocytes are enlarged and with inclusions in the swollen nuclei. The astrocytes are also affected and resemble transformed cells in glioblastoma. The central part of the lesions can exhibit necrosis with phagocytosis (Shah 1996). Before the AIDS epidemic PML was a rare disease occurring in leukemia and lymphoma patients. However, after the onset of the AIDS epidemic the incidence has increased, since 3-8% of HIV positive patients develops PML when the T-cell count becomes low (Berger et al. 1999). An additional factor for the development of PML in HIV positive patients has been suggested to be a specific interaction between HIV-1 and JCV, via the HIV-1 regulatory protein Tat (Tada et al. 1990; Major et al. 1992; Gallia et al. 1998; Gordon et al. 1998; Gordon et al. 1998). The median survival of untreated PML is less than 6 months (Berger 2000). However, after the introduction of highly active anti-retroviral therapy (HAART), for review see Flepp et al. 2001, about 7-9% of the patients will have a prolonged survival, but still less than 12 months (Berger 2000). Antiviral treatment with cytarabine has not been successful (Berger 2000).

3.7.4 Malignant mesothelioma and SV40

MM are tumors of the serosal membranes such as pleura, peritoneum and pericard, where pleura is the most common localization, for review see Dejmek 1994. MM has mainly been associated to a heavy exposition of asbestos with a latency period of about 20-35 years after exposure, and is most commonly observed in males above the age of 50 years. The incidence in Sweden was in 1990 11/million (Cancer incidence in Sweden 1990). The incidence of MM has risen dramatically the last 20 years due to the increased industrial use of asbestos since World War II (Fisher et al. 1999) Nevertheless, 10% of individuals with prolonged and heavy exposure to asbestos develop MM, while 20% of all MM patients lack a history of asbestos exposition, indicating that additional factors may be involved in the development of MM (Roggli et al. 1992).

In 1994 Carbone *et al.* reported that SV40 like DNA sequences were present in 60% of the investigated MM samples from USA (Carbone et al. 1994). This findings has posed a great deal of concern, since SV40 has been distributed

randomly to millions of people between 1955 and 1963 in the USA and Europe, through polio vaccine produced in SV40 infected monkey kidney cells (Shah et al. 1976). In addition, SV40 DNA-like sequences have been detected not only in MM but also in other human tumors, such as brain and bone tumors (Butel et al. 1999; Carbone et al. 1999).

Additional studies have confirmed the presence of SV40 like sequences in 41-86% of MM material from Italy, the UK, Germany and the USA (Pepper et al. 1996; De Luca et al. 1997; Dhaene et al. 1999; Cristaudo et al. 2000; Shivapurkar et al. 2000; Strizzi et al. 2000). Subsequently, SV40 suggested to induce tumors, by binding of its large T-antigen to and inactivation of p53, Rb and related proteins (Butel et al. 1999; Carbone et al. 1999), and through the induction of chromosomal aberrations (Carbone et al. 1999), is now suspected to be a factor or a co-factor for the development of MM (Carbone et al. 1997; Wiman et al. 1997). However presence of SV40 has not been confirmed in MM from Turkey and Finland, or in other studies from the UK and the USA (Strickler et al. 1996; Hirvonen et al. 1999; Mulatero et al. 1999; Emri et al. 2000).

In Sweden national polio vaccination started in 1957 and approximately 700 000 individuals, mainly pre-school and school children born between 1946 and 1953 may have potentially been exposed to SV40 via contaminated polio vaccine. From 1958, a Swedish inactivated polio vaccine was used exclusively (Olin et al. 1998). This vaccine was produced in kidney cells from Javanese macaques, suggested to be free of SV40 and presumably from 1958, the risk of exposure to SV40 in Sweden should therefore have been low or neglectable (Olin et al. 1998).

4. Materials and Methods

MATERIALS

4.1 Sera from BMT patients and their donors

(Paper I)

Sera were collected from 45 BMT patients, 7 months to 17 years of age, transplanted at Huddinge University Hospital between 1987-1996, as well as sera from their donors, 2 to 49 years of age. Nine patients had documented episodes of HC and 3 patients had hematuria without cystitis symptoms. Two to 5 samples from 45 BMT patients and one sample from each donor were collected, totally 204 samples. One sample was collected before BMT, and variable amount of samples after BMT, if possible at 3, 6 and 12 months after BMT. Samples were stored at –20ºC and preheated at 56ºC for 30 min before testing.

4.2 Urine samples from BMT patients

(Paper II and III)

Urine samples from 3 autologous and 22 allogenic BMT patients transplanted at Huddinge University Hospital were collected between 1993-1998. Seventeen urine samples were obtained from 16 patients with HC during HC episodes, and 15 urine samples were collected from 9 patients without HC. One to 4 samples were collected from each patient, 1 day to 4 months after BMT and stored at –20ºC or –70ºC for 5 months - 6 years. In summary 31 samples were collected from 25 patients, and 2.5µl, 5µl and 10µl from each urine sample were tested directly by PCR.

4.3 Urine samples from RTX patients

(Paper IV)

Urine samples from 170 RTX patients, 66 patients with a living donor (LD) graft and 104 with a cadaver donor (CD) graft, transplanted at Huddinge University Hospital were collected between 1995-1998. The majority of the

samples were tested within 48 h. after collection, but some were stored at –20ºC and analyzed within a week. Totally 383 urine samples were collected. In PCR 2.5µl, 5µl and 10µl from each urine sample were used.

4.4 CSF samples

(Paper V)

CSF samples from different departments at Huddinge University Hospital were collected from patients with herpes simplex type 1 encephalitis (43 samples from 39 patients), enteroviral meningitis (20 samples from 20 patients), nonenteroviral meningitis (15 samples from 15 patients) and from MS-patients (58 centrifuged samples from 45 patients, 45 samples during exacerbation, and 13 samples during remission). Four JCV positive CSF samples from four AIDS patients with PML from an earlier study were used as positive controls (Hammarin et al. 1996). The samples were stored at –20 ºC or –70 ºC before testing and were pre-heated at 95ºC for 10 min before PCR. Totally 140 CSF samples from 123 patients were collected, and 10 µl of each samples were tested by PCR.

4.5 Malignant mesothelioma and adenocarcinoma tissue

(Paper VI)

Formalin-fixed and paraffin-embedded tissue sections from 41 pleural mesothelioma, 5 pleural adeno carcinomas from patients (born 1893-1958 and diagnosed 1978 – 1994) and mesothelial tissue were obtained from the Swedish Mesothelioma Bank, Dept. of Pathology at Huddinge University Hospital. Three sections/sample 10 µm thick were collected at two different occasions, to ensure enough material. An adjacent section was examined microscopically to ensure presence of tumor tissue in the PCR sample.

4.6 Serology methods

(Paper I)

4.6.1 Hemagglutination inhibition assay (HAI)

The HAI –tests were performed in microtiter plates (Nunc Microwell, Nunc, Roskilde, Denmank). The serum samples were diluted in two-fold dilutions from 1:10 to 1:10240. A mix of 25µl serum and 25µl BKV antigen, corresponding to 4 HA units of sonicated BKV cultured in Vero cells, were incubated at 37ºC for 30 min. Then 50µl of 0.5% human group 0 erythrocyte solution was added, and the plates were incubated in +4ºC for 4 h. For each HAI test seronegative human sera or fetal calf sera were used as negative controls. Two seropositive volunteers were used as positive controls. Control for viral antigen, red blood cells and each sera sample were included as well. The HAI titer of the sera corresponded to the highest dilution of the sera that completely inhibited hemagglutination. Titers of 1:160 were regarded as positive for BKV. A four-fold or higher increase were judged as a significant titer increase.

Sera were also pre-treated with receptor destroying enzyme (RDE, Bio Whittakers), to remove non-specific inhibitors (Gardner et al. 1971; Flaegstad et al. 1986). When we used RDE a titer of 1:80 was considered as BKV positive. The RDE-HAI titer was regarded as final if a discrepancy between HAI with and without RDE, was observed.

4.6.2 BKV IgG and IgM-class specific Enzyme linked immuno-sorbent assay (ELISA)

The Immunoglobuline G (Ig G) and Immunoglobuline M (Ig M) ELISA used were solid phase Ig G ELISA (SPELISA) developed and described earlier by (Flaegstad et al. 1985). The tests were performed by Dr Flaegstad at Tromsö University.

4.7 Sequencing

(Paper II)

4.7.1 BKV NCCR nested PCR

In order to obtain a double stranded amplificate of the NCCR fragment, we used a nested PCR with the outer primers BKTT-1 and BKTT-10 in the first round and the inner primers BKPA-1 and BKTT4 in the second round (Table 1.) (Sundsfjord et al. 1990), Correspondance: P. A. Andresen, accession no. J02038 K00058 V01108 V01110). For both rounds the samples were preheated at 94ºC for 5 min. The PCR mixture for each reaction contained PCR buffer including $MgCl_2$ (PE Biosystem), 0.48mM dNTP (equal mix), 50pmol each of the outer primers, 2U Taq, 10µl of the pretreated sample and double distilled (dd) water equaling a total volume of 100µl. The samples were subjected to 40 cycles of 1 min at 94ºC, 1 min at 55ºC and 2 min at 72ºC, and then finally one cycle of 7 min at 72ºC. Five µl of the product from the first 40 cycles was transferred to an identical PCR mixture with the exception of an exchange of primers to BKPA-1 and BKTT-4 same concentration as above and dd water up to 100µl.

4.7.2 BKV VP1 region single PCR.

In order to amplify a double stranded amplificate of the VP1 region we used a single PCR with the primers 327-1 and 327-2 (Table 1) (Jin et al. 1993). Before PCR the samples were preheated at 94ºC for 2 min. The PCR mixture for each reaction contained PCR buffer (PE Biosystem), 0.2mM $MgCl_2$, 0.8mM dNTP, 100pmol of each primer 2U Taq, 10µl of the sample and dd water equaling a total volume of 100µl. The samples were subjected to 35 cycles of 1 min at 91ºC, 1 min at 55ºC and 1 min at 72ºC, and then finally one cycle of 4 min at 72º C.

4.7.3 Big Dye PCR before sequencing

Fifty µl of either NCCR or VP1 PCR products were purified on Pharmacia S-400 HR Microspin columns at 735xg for 2 min. The concentration of DNA was estimated by spectrophotometry. Single stranded marked DNA necessary

for the sequencing procedure was produced by a Big Dye PCR (ABI Prism®). Each sample included 8 µl of Terminator Ready Reaction (TRR) mixture (ABI Prism®), 3.2 pmol of primer BKPA-1 for NCCR or 327–1 for VP1, 100ng of template/PCR product and dd water was added to yield a final volume of 20 µl. The samples were subjected to 25 cycles of 30 sec at 96ºC, 15 sec at 50ºC, 4 min at 60ºC.

4.7.4 Sequencing electrophoresis of Big Dye PCR products.

To 20µl of each PCR product (NCCR and VP1) 2µl 3M NaAc (pH 4.6) and 50µl of 95% EtOH at room temperature were added. The samples were vortexed and left in room temperature for 15 min and then spun for 30 min at 13 000 r/min. The supernatants were replaced by 250µl of 70% EtOH at room temperature and vortexed, then spun for 10 min at 13 000 r/min. After disposal of the supernatant the samples were dried in a vacuum centrifuge for 3 min. Twenty µl of Template Suppression Reagent (TSR) sample mix (ABI Prism®) was then added to each dried sample. Following incubation on a shaker for 10 min, the tubes were vortexed briefly and subsequently heated for 2 min at 95^0 C and then finally chilled on ice. The samples were then transferred to new tubes and covered with a tubsepta and analyzed in an ABI Prism 310 Genetic Analyzer®. The obtained NCCR and VP1 sequences were aligned using the DNASIS® Sequencing Analysis Software. In addition, the NCCR and the VP1 sequences were compared with those available at the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/).

4.8 Real Time PCR

(Paper III)

For Real-Time quantitative PCR analysis we used an ABI PRISM 7700 Sequence Detector© (Applied Biosystem, Foster City, USA) (Heid et al. 1996). Each 25µl of PCR reaction contained 2µl of template, Taqman Buffer PCR©, 0.8mM dNTPs (equal mix), 2mM $MgCl_2$, 7.5pmol BK-S, 7.5pmol BK-AS, 7.5pmol BK-Probe (Table 1), 1.25U Taq Gold DNA polymerase© and dd water. The BK-Probe and thereby the BK-S and BK-AS primers were selected

from the early region. All samples were analyzed in a 96 well plate (Applied Biosystem®) covered with optic caps. The samples were subjected to 50°C for 2 min, 95°C for 10 min, for 40 cycles involving denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Fluorescence was measured automatically during each PCR cycle through the entire amplification period. The first PCR cycle when Taqman© software detected a fluorescence increase associated with an exponential growth of a specific PCR product was defined as the threshold cycle (C_T). The more template DNA in the sample from start, the lower C_T-value (Gibson et al. 1996; Heid et al. 1996).

Development of a standard DNA for Real-Time PCR.

For each reaction we used PCR Taqman buffer©, 1.5mM $MgCl_2$, 20pmol BK-Sense primer (BK-S), 20pmol BK-Antisense primer (BK-AS), 0.6 mM dNTPs (equal mix), 3 U Taq Gold DNA polymerase©, double distilled (dd) water and 10 µl of BKV control, totally 50µl. Amplification consisted of an initial step of 96°C for 2 min, followed by 30 cycles of 92°C for 30 s, 60°C for 30 s, 72°C for 30 s, and ended with 72°C for 5 min. The 272 base pairs (bp) long amplicon was extracted from a 2% agarose gel with a QIAquick Gel Extraction Kit© (Qiagen® Helden, Germany), ligated into a pGEM-T Easy Vector©, transformed into competent cells (JM 109©, Promega® Madison, USA) and cultivated on LB-Ampicillin agar plates (100 µg/ml) pre-treated with IPTG and X-Gal. Five plasmid positive colonies were selected and re-cultivated. Plasmids were extracted with QIAprep Spin Miniprep kit© (Qiagen®), and sequenced in an ABI PRISM 310© (Applied Biosystem®) with BK-S and BK-AS as primers using Big Dye© reagents described earlier (Priftakis et al. 2001), to confirm the sequence of the 272 bp amplicon. The amplicon was compared to sequences available at GenBank, matching BKV Accession No: NC 001538, JO 2038, VO 1110, VO 1108. Finally, the concentration was measured by optical density (Lambda Polynom UV-1601©, Shimadzu®), and the plasmid copy number per litre was calculated, according to the following formula: (Plasmid concentration (g/l) x 6.02 x 10^{23})/

((Plasmid + Insert (bp)) x 660) (Whitten 2000). Serial dilutions containing $9.6x10^4$, $4.8x10^4$, $9.6x10^3$, $4.8x10^3$, $9.6x10^2$, 9.6x10 plasmids/2µl were prepared.

4.9 Human polyomavirus nested PCR

(Paper IV – VI)

Presence of BKV and JCV DNA was analysed by a nested PCR with primers P1-P4 (Table 1) described earlier (Bogdanovic et al. 1994). An optimised reaction mix contained 50mM NaCl, 10mM Tris-HCl (pH 8.3), 4mM $MgCl_2$, 400µM of each dNTP, 0.0625µM each of the outer primers P3 and P4 (Table 1.) and 1U of Taq polymerase©, sample and dd water to a total amount of 50µl. From the first amplification 2,5µl of product were transferred to the second PCR mixture containing 50mM NaCl, 10mM Tris-HCl (pH 8.3), 6mM $MgCl_2$, 50µM of each dNTP, 0.17µM each of the inner primers P1 and P2, 1U of Taq polymerase© and dd water to a final volume of 50µl. The samples were subjected to an initial step of 95°C for 5 min, then 94°C for 1 min, 60°C for 1 min 30s and 72°C for 1 min and 30 s. Twenty cycles were performed for the first round and 40 cycles for the second. The products were electrophoresed in a 2% agarose gel. The 176 bp BKV product and the 173 bp JCV product were discriminated by enzymatic cleavage by Bam H1 or Hinf 1.

4.10 SV40 specific single PCR

(Paper VI)

An SV40 specific PCR with the SV.for3 and SV.rev primers, amplifying a 105 bp product in the Rb, p107 and RB2/p130 binding domain of the large T antigen region of the SV40 was used (Bergsagel et al. 1992). Ten µl of each sample containing 50 – 1000ng of DNA was added to 40µl of a PCR reaction mixture of 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 200µM of each dNTP, 50µM of each primer (Table. 1) and 1.25U of Taq polymerase© (Strickler et al. 1996). Samples were subjected to an initial step of 94°C for 10 min, 40 cycles of one minute each of 94°C, 52°C and 72°C, and finally 72°C for 10 min (Strickler et al. 1996).

4.11 HLA, β-globin and ribosomal S14 PCR

(Paper VI)

Extracted DNA from all tissue samples was tested by PCR using primers for either the HLA DQ locus (Saiki et al. 1986), the β-globin gene (Saiki et al. 1986) or the ribosomal S14 gene (Rhoads et al. 1986). Only samples that could be amplified with at least one of these PCRs, and thus confirmed amplifiable, were included in our study.

4.12 DNA extraction

(Paper VI)

All samples were deparaffinized by adding Xylene© (Merck®, Darmstadt, Germany) and then washed with EtOH (95%) 2-3 times. The dried pellet was re-suspended in Proteinase K digestion buffer or ATL-buffer (QIAamp® DNA Mini Kit, Qiagen™, Hilden, Germany), and digested with Proteinase K. For the human polyoma virus nested PCR, DNA was extracted by a Phenol (Sigma™, Steinheim, Germany) – Chlorophorm (Merck™) 1:1 mixture, followed by ethanol precipitation. For the SV40 specific PCR, DNA was extracted by a "QIAamp® DNA Mini Kit and a QIAampDNA Blood Mini Kit" (Qiagen™, Hilden, Germany).

TABLE 1. Sequence of primers and probe used in PCR

Name	PCR type	Sequence 5' - 3'	Virus	Region
BK-S	Real Time	GCAATCTATCCAAACCAAGGGCTCTT	BKV	LT
BK-AS	Real Time	GGGGCGACGAGGATAAAATGAAGA	BKV	LT
BK-Probe	Real Time	TTTTTGGAACAAATAGGCCATTCCTTGC	BKV	LT
P1	HuPy	AGTCTTTAGGGTCTTCTACC	BKV/JCV/SV40	LT
P2	HuPy	GGTGCCAACCTATGGAACAG	BKV/JCV/SV40	LT
P3	HuPy	GTATACACAGCAAAGGAAGC	BKV/JCV/SV40	LT
P4	HuPy	GCTCATCAGCCTGATTTTGG	BKV/JCV/SV40	LT
BKTT-1	NCCR	AAGGTCCATGAGCTCCATGGATTCTTCC	BKV	NCCR
BKTT-10	NCCR	CCAGGTTTTACCAACTTTCACTGAAG	BKV	NCCR
BKPA-1	NCCR	TACTACTTGAGAGAAAGGGTGGA	BKV	NCCR
BKTT-4	NCCR	ACAAGGCCAAGATTCCTAGGCTCGC	BKV	NCCR
327-1	VP-1	CAAGTGCCAAAACTACTAAT	BKV	VP-1
327-2	VP-1	TGCATGAAGGTTAAGCATGC	BKV	VP-1
SV.for3	SV40	TGAGGCTACTGCTGACTCTCAACAC	SV40	LT
SV.rev	SV40	GCATGACTCAAAAAACTTAGCAATTCTG	SV40	LT

4.13 General considerations

► In order to avoid PCR contamination, recommended precautions were undertaken as well as running all samples in duplicates and using negative water samples between each sample.

► One of the advantages with the Real Time quantitative PCR was that the tube/well with amplified material was never reopened and read optically through the optical cap. In this way the risk of contamination of amplified material is minimized.

► The higher prevalence of positive HAI titers compared to ELISA, could be due to that HAI measures both IgG and IgM.

► We found it difficult to extract DNA from the paraffin embedded samples, and the amount of DNA was generally small. Furthermore, the quality of the extracted DNA was generally low, which made it very difficult to sequence. The main difference between the two extraction methods was that it was easier to lose material when performing the phenol – chlorophorm extraction, compared to the Qiagen-kit. The frequency of successful extraction was the same for both methods.

5. Results and Discussion

5.1 Paper I

Primary BK virus (BKV) infection due to possible BKV transmission during bone marrow transplantation BMT is not the major cause of hemorrhagic cystitis in transplanted children.

The aim of this study was to investigate if primary BKV infection, possibly transmitted from donor to recipient during allogenic BMT in children with leukaemia, was associated with the occurrence of HC.

To investigate if HC was associated to a primary BKV infection during BMT, we focused on BMT children, since they would more likely be BKV seronegative (Gardner 1973; Shah et al. 1973; Taguchi et al. 1982; Flaegstad et al. 1986) (Fig 10). Serum samples from 45 BMT recipients (7 months to 17 years) with and without HC/hematuria and their donors (2 to 49 years) were collected before BMT, and from recipients 3, 6 and 12 months after BMT. The samples were analyzed for BKV antibody titers, by both a BKV specific hemagglutination inhibition (HAI) test and a BKV specific enzyme linked immunosorbent assay (ELISA). In parallel to this, patients' records were investigated for occurrence of HC as well as for intravenous immunoglobuline (IVIG) infusions, in order to exclude false positive results. Nine patients had clinical manifestations of HC, defined as hematuria and cystitis symptoms and three patients had asymptomatic hematuria. The 9 patients with HC had an onset of symptoms 5-8 weeks after BMT, defined as late HC (see 3.7.1) and with a duration between 1 to 8 months.

Before BMT all patients and 44/45 (98%) donors were HAI positive and 39/45 (87%) patients and 38/45 (84%) donors were ELISA positive. These results were somewhat unexpected since primary BKV infection usually occurs at the age of 2 – 5 years and BKV seroprevalence in adults is 70-80% (Gardner 1973; Shah et al. 1973; Taguchi et al. 1982; Flaegstad et al. 1986). The age distribution of BKV seropositive patients in our study was thus more similar to that reported for children with cancer (Flaegstad et al. 1988). One explanation

is that these patients received blood transfusions during their treatment, and thereby were infected earlier compared to healthy individuals. However, since there was no difference between the patients and their donors with regard to a high BKV seroprevalence, this explanation may not be relevant.

The HC/hematuria patients were all HAI seropositive before BMT, and 11/12 (91%) were IgG ELISA positive. After BMT 7/12 (58%) of the HC/hematuria patients presented serological changes such as an IgM positive ELISA or a significant HAI titer increase or both. However, sera were only available in 7/9 patients at the onset of HC symptoms. Only one patient had a significant HAI titer increase during HC symptoms, and this patient had not received any IVIG. Four patients showed IgM or HAI titer increase 6 – 12 weeks after the HC symptoms had resolved. All but 1 of the HC patients donors, were seropositive by HAI, and 6/9 (67%) by IgG ELISA.

All patients without HC were HAI seropositive before BMT and 28/33 (85%) were IgG ELISA positive. After BMT 7/33 (24%) had serological changes such as a positive IgM ELISA, a significant HAI titer increase or both. All non-HC patient donors were HAI positive and 11/12 (91%) were IgG ELISA positive.

In conclusion primary BKV infection was not the major cause of HC. In fact, it was not possible to investigate if BKV primary infection was the cause of HC, since all patients with HC were seropositive before BMT. Nevertheless, the HC/hematuria patients had more serological changes (58%) such as positive Ig M ELISA, significant HAI titer increase or both, compared to non-HC patients (24%) ($p=0.017$). Furthermore, the donors of non-HC patients (91%) were more frequently IgG ELISA seropositive compared to HC patients (67%). This difference was not significant ($p= 0.053$), but could imply that a high anti-BKV titer in the donor decreases the risk for development of HC in the recipient.

5.2 Paper II and III

Studies on BKV subtypes and/or mutations, in BMT patients with hemorrhagic cystitis

The aims of these studies were to investigate a) if different strains of or mutations in BKV were responsible for the development of HC and b) if these mutations induced an increased BKV excretion in the urine.

Urine samples previously confirmed to be BKV DNA positive, were collected from 25 BMT patients both with and without HC. To identify possible BKV mutations in these samples, the BKV NCCR and the BKV VP1 encoding region were sequenced. When sequencing the NCCR, we found C to G mutations within the Sp1 binding site. These mutations were present in 7/16 (43%) patients with HC, but were not found in any of the patients without HC ($p=0.019$). The C to G mutation was located at position 249 in 6 cases and at position 251 in one case. In addition, C to T mutations at position 254, were found in a similar proportion in patients with HC (6/16) (38%) and without HC (4/9) (44%). Also other point mutations were found within the NCCR, but these were located outside the binding sites for other transcriptional factors. Further analyzes of the NCCR showed the archetypal organization, with intact O, P, Q, R and S blocks, in the BKV of all patients. This was not an unexpected finding since the NCCR of BKV isolated from urine usually shows an archetypal organization (Negrini et al. 1991), while the BKV NCCR is often rearranged after repeated passages in cell cultures (Rubinstein et al. 1987; Azzi et al. 1996; Jin et al. 1996). Nevertheless, one study has shown a rearranged NCCR in BKV isolated directly from PBMC (Degener et al. 1999). The VP1 encoding region was successfully sequenced in totally 21 patients, and no differences between patients with and without HC were found. The WW and WWT variants dominated both in patients with and without HC, which was similar to previous studies in immunosuppressed patients and healthy individuals (Arthur et al. 1989; Negrini et al. 1991; Markowitz et al. 1993).

In order to further investigate the clinical relevance of the detected mutations in the NCCR Sp1 binding site, previously suggested to be a positive regulator for BKV transcription (Cassill et al. 1989; Sundsfjord et al. 1990), we developed a quantitative RealTime PCR. Samples from 21 of the 25 BKV

positive BMT patients were still available and totally 18 samples were successfully analyzed, 12 from patients with HC (6 with and 6 without the BKV Sp1 binding site mutation) and 6 patients without HC. The urine samples from HC patients with BKV Sp1 binding site mutations, had a median between 11–15x10^5 BKV copies/µl, as compared to a median between 2.5x10^4–1x10^5 BKV copies/µl in samples from HC patients without Sp1 site mutations. Urine samples from non-HC patients had a median between 4–5x10^3 BKV copies/µl. Furthermore, while 50% of the samples in the HC groups expressed 1x10^6 copies/µl or more only one of the samples in the non-HC group contained a virus quantity higher than 5x10^5 copies. A Mann-Whitney test was performed to determine possible significant differences in BKV copy number between patient groups. A tendency of increased BKV excretion in BMT patients with HC ($p=0.0752$) was observed when compared to non-HC patients. When the groups were analyzed separately this tendency was still apparent in HC patients with BKV Sp1 site mutations ($p=0.0782$), but not in HC patients without mutations ($p=0.2002$). Nevertheless, there were no significant differences between the two HC groups ($p=0.4712$). However, we want to point out that the insignificant difference HC and non-HC patients was due to only one patient in the non-HC group who excreted above 10^6 copies/µl. When this patient was excluded a significant difference was obtained between non-HC patients and HC patients with Sp1 site mutations ($p=0.0176$), as well as between non-HC and HC patients without these mutations ($p=0.0446$). It should be mentioned that the indicated patient had a mild GVHD and cystitis symptoms, without detectable hematuria, and was thus not defined as an HC patient.

In conclusion, BKV with C to G mutations in the Sp1 binding site were shown to be significantly over represented in HC patients. However it could not be statistically confirmed that BKV with Sp1 site mutations induced a higher viral load in patients with HC compared to patients without HC. However, the use of a BKV Real-Time quantitative PCR offers valuable information when analyzing urine samples from BMT patients. Our data suggest that levels of BKV above 10^4 BKV copies/µl indicate a risk for HC. Nevertheless, since broad variations exist in BKV excretion between individual patients independent of HC status, this threshold should be used with caution.

5.3 Paper IV

Polyomaviruria in renal transplant patients is not correlated to the cold ischemia period or to rejection episodes.

The aim of this study was to investigate if BKV/JCV reactivation in RTX patients is influenced by the graft cold ischemia time, and if viral reactivation resulted in a higher frequency of graft rejection episodes.

Reactivation of polyomavirus has been observed after occlusion of the renal artery in mice (Atencio et al. 1993). Correspondingly it has been suggested that JCV and /or BKV is reactivated in the kidneys after ischemia (Coleman et al. 1973; Coleman et al. 1978). However, this has not been studied extensively or been correlated to graft ischemia time which varies between grafts from LD (30 min) and CD (12 – 24h.). To further study this issue 383 urine samples were obtained collected from 170 (104 CD and 66 LD) patients between 1995-1998 from Dept. of Transplant surgery at Huddinge University Hospital Transplantation unit. Samples were collected from 1 week to two years after transplantation and were analyzed for the presence of polyomaviruses with a human polyomavirus nested PCR. Patient records were investigated for registered complications after RTX. Out of 170 patients 55 (33%) were found to be positive for human polyomavirus in their urine samples at some time point after transplantation. There was no significant difference in excretion of human polyomaviruses between recipients of LD kidney grafts (36.5%) vs. CD kidney grafts (30%). In 71 (42%) out of 170 patients one or more graft rejection episodes were observed according to the data from patients' records. Of the 71 patients with rejection episodes, 19 (27%) excreted polyomaviruses, while in the group of 99 patients without rejection episodes 36 (36%) had polyomaviruria. It could be argued that the higher rate of polyomaviruria in patients without rejection episodes, could be due to a higher immunosuppression. However, the difference between the two groups was not significant.

In summary the prevalence of BK and JC viruria was similar for patients with LD and CD grafts and thus, independent of graft ischemia time. Further more, we found no correlation between polyomaviruria and graft rejection episodes during RTX.

5.4 Paper V

JCV DNA detected by PCR in CSF samples is diagnostic for PML

The aim of this study was to investigate if detection of JCV DNA in CSF by PCR is indicative for PML, and specifically exclude that JCV is reactivated during other viral infections in the CNS or during MS.

In this study we collected CSF samples from 39 patients with HSV-1 encephalitis, 20 patients with enteroviral meningitis, 15 patients with nonenteroviral meningitis as well as 45 MS patients. The samples were analyzed for presence of JCV DNA with a human polyomavirus nested PCR. Four CSF samples positive for JCV, from AIDS patients with a PML diagnosis, were included as positive controls. We detected no JCV DNA in the samples from patients with HSV-1 encephalitis, enteroviral meningitis, nonenteroviral meningitis or MS patients. However, as expected JCV DNA was detected in all 4 samples from the AIDS patients with a histopathlogic diagnosis for PML.

In conclusion detection of JCV DNA in the CSF by PCR is further confirmed to be indicative for PML, and is not detected in patients with HSV-1 encephalitis, enteroviral meningitis, nonenteroviral meningitis or during MS.

5.5 Paper VI

Presence of Simian Virus (SV40) is not a frequent finding in Swedish malignant mesothelioma.

The aim of this study was to investigate if SV40 or human polyomaviruses were present in Swedish malignant mesothelioma and further to investigate if there was a correlation to the administration of SV40 contaminated polio vaccine in these patients.

In this study 41 paraffin-embedded pleural MM samples and 5 adenocarcinomas were analyzed for the presence of SV40, BKV or JCV DNA with a "human" polyomavirus nested PCR. Additionally presence of SV40 was analyzed with a SV40 specific single PCR and confirmed by sequencing.

Of the 41 MM samples, 30 could be extracted and amplified successfully, 14 were positive by HLA DQ PCR, one by β-globin PCR, and 15 by S14 PCR. Three (10%) of the 30 MM samples, were SV40 positive both by the "human" polyomavirus nested PCR and the SV40 specific PCR. In addition, one sample was SV40 positive only by the human polyoma nested PCR and another sample was positive in only by the SV40 specific PCR. The presence of SV40 DNA could only be confirmed by sequencing in the three samples positive with both PCR methods. Consequently, only these samples were considered as truly SV40 positive. All control samples were negative for SV40.

The three SV40 containing tumor samples were derived from three patients born in Sweden between 1909 and 1929. The two additional samples, positive only by one method and not possible to confirm by sequencing, originated from patients born 1905 and 1934. The remaining SV40 PCR negative tumor samples were derived from patients born 1893 - 1958. Thus, the SV40 frequency in Swedish MM samples is far below that reported (40-70%) in many studies from the USA, Italy, UK and Germany (Pepper et al. 1996; De Luca et al. 1997; Dhaene et al. 1999; Cristaudo et al. 2000; Shivapurkar et al. 2000; Strizzi et al. 2000), but more similar to other reports from Turkey, Finland, UK and USA (Strickler et al. 1996; Hirvonen et al. 1999; Mulatero et al. 1999; Emri et al. 2000). One explanation for the incoherent reports regarding the presence of SV40 in MM is that SV40 contaminated polio vaccine was distributed unevenly both within the USA and worldwide (Carbone et al. 1996). Other suggestions have been differences in the

sensitivity of the methods used and laboratory contaminations (Strickler et al. 1996; Griffiths et al. 1998).

The reasons for the similarity between the Swedish and Finish MM findings could be that only few SV40 contaminated polio vaccine batches were distributed to Scandinavia and that Finland and Sweden produced SV40 free polio vaccine from 1958 (Olin et al. 1998). These findings comply with a study on Turkish MM, where the absence of SV40 DNA was explained by the late start of polio vaccination, with SV40 free vaccine, in the 1970s (Emri et al. 2000).

In conclusion, the frequency of SV40 in Swedish pleural MM is low and we suggest that this is due to that SV40 contaminated polio vaccine not was distributed widely in Sweden. The three MM SV40 positive patients born 1909-1929 may have been vaccinated with the first batches of potentially SV40 contaminated polio vaccine.

6. Conclusions

► Primary BKV infection due to possible BKV transmission from the donor to the recipient during BMT is not the major cause of HC in allogenic BMT children.

► BKV with C to G mutations in the NCCR Sp1 binding site is significantly over represented in BMT patients with HC and absent in without HC.

► It could not be statistically shown that BMT patients with HC, excreting BKV with C to G mutations in the NCCR Sp1 binding site, had a higher BK viral load in the urine, as compared to patients without HC.

► The prevalence of BK and JC viruria is similar for RTX patients with LD and CD grafts and thus independent of graft cold ischemia time. Furthermore, no correlation between polyomaviruria and graft rejection episodes was observed.

► Detection of JCV by PCR in the CSF is indicative for PML, and JCV is not detected in the CSF of patients with HSV-1 encephalitis, enteroviral meningitis, non-enteroviral meningitis or during MS.

► The frequency of SV40 in MM from Swedish patients is low, which indicates that SV40 contaminated polio vaccine not was distributed widely in Sweden even before 1958.

Acknowledgements

I want to express my sincere gratitude to all the people who have supported me in all various ways during my studies:

To my supervisor Tina Dalianis for without hesitation (?) dragging me into research, for always sharing your energy and enthusiasm for science, for your scientific guidance, for always being available, for you ability to sketch out the lines and for always using humor as a tool in the everyday work…

To my co-supervisor Gordana Bogdanovic for teaching me all "hands on work" in the lab, for your patience when things not always became right in the beginning, for your ability to see the important details and for your overwhelming generosity…

You both for guiding and helping me with the project, being supportive colleagues and friends, giving advice about career and how and how not to combine clinic, research and the life outside...

To Professor Stefan Einhorn director at Department of Oncology-Pathology for enable me to work at the department, for encouragement and for good collaboration with "OPUS".

To Professor Andres Vahlne at the Division of Clinical Virology at Huddinge University Hospital, where I started my PhD studies.

To Rolf Kiessling, Pavel Pisa, Håkan Mellstedt and Hodjatt Rabbani for support and good advice.

My deepest respect to my co-authors for your professional contributions to our work.

To all PhD students, postdocs and staff at IGT, for creating a creative, professional, international atmosphere at "floor 1" at CCK … and for all nice chats at "lunchrummet". Also to all other PhD students, as well as everyone else at CCK for being professional, helpful and creating a very pleasant atmosphere.

To all "old friends", the PhD students and staff at Clinical Virology, for support and having fun during the years at Clinical Virology, Huddinge University Hospital

To my "comrades in arms" in the group: Shirin for making things more fun at the lab, and for coming with inspiring "crazy ideas", Hanna for sharing the good and bad being a med.student and PhD. student, Andrea for also being a half-greek in the "diaspora", Zsofia for being my first "teacher" at the lab, Daniela for good advice and support and for company in the early morning hours, Ilona for good advice and for inviting me to my first dissertation party ever!, Torbjörn for good advice and for making the minority in the group grow by 100%, Liselotte for good support and being the first in the row of next generation PhD students, Karin for being our latest recruitment, Sofia, Kalle

and all other students spending some time in the group and who hopefully will join the group later. You all have been a great support.

To the ”OPUS” committee for fun and good collaboration: Dan Grandér, Evi Gustavson-Kadaka, Riitta Perttunen-Elo, and Anna Bergsmedh

To my friends in “Stockholms Arbetarekommun” for reminding me that there are still so many things to change in this world…

To my friends, no one forgotten, for sharing my joys, sorrows and dreams.

To my “mecenater” for funding me and studies: The Tobias Foundation, The Swedish Cancer Foundation, The Children Cancer Foundation, The Karolinska Institute and Stockholm County Council.

To my family:

My mother for being my mother, always loving me, always supporting me, always believing in me and for proving to me that everything in life is possible as long as you fight hard.

All my ”mostrar”: Ingrid, Karin, Margareta, Stina, Kerstin, their families and my other relatives in Jönköping, for spoiling me and for their great support all my life.

Panayiotis, Maria, Katerina, Dimitris and my other relatives in Greece, for always making me feel at home in Greece, for have been trying to make me a bit more “Greek” and for make me remember where a part of me belong.

Pilar, Amanda, Gudrun (and Gurke) for receiving me with open arms (and open paws).

And last but most important, to my beloved Elisa for your never-ending support and for sharing the most important in life with me: our love and our future! Without you this hadn’t been possible…

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