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RESEARCH ARTICLE

Low Frequency of SV40, JC and BK Polyomavirus Sequences in Human Medulloblastomas, Meningiomas and Ependymomas

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Several reports have suggested a role for polyomaviruses in the pathogenesis of human brain tumors. This potential involvement is not conclusively resolved. For the present study, a highly sensitive PCR-assay with fluorescence-labelled primers was developed to search for polyomavirus sequences in human brain tumor and control DNA samples. The assay was shown to detect approximately one viral large T-antigen (TAg) gene per 250 cells. We identified simian virus 40 (SV40)-like sequences in 2/116 medulloblastomas, in 1/131 meningiomas, in 1/25 ependymomas and in 1/2 subependymomas. A single case of ependymoma contained SV40 VP-1 late gene sequences. Moreover, one of the meningioma samples showed JC virus sequences. In contrast, 60 hepatoblastoma samples and 31 brain samples from schizophrenic patients were consistently negative. BK virus sequences were not detectable in any of our samples. Immunohistochemical analysis of two SV40 positive tumor biopsies failed to detect large TAg in the tumor cells. In the JC positive meningioma, immunoreactivity for the viral late gene product (VP-1) was not observed. Our data do not entirely rule out SV40 and JC virus as an initiative agent with a hit-and-run mechanism. However the low frequency of virus sequences and the absence of TAg protein expression argue against a major role of these viruses in the pathogenesis of human medulloblastomas, meningiomas and ependymomas.

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Introduction

Since it has been recognized that poliovirus vaccines were contaminated with live simian virus 40 (SV40) between 1955 and 1963 there is an ongoing debate about the potential involvement of polyomaviruses in the development of human cancers (6, 10, 41, 50). The macaque polyomavirus SV40 as well as the human polyomaviruses JC and BK all display a strong neurooncogenic potential after inoculation into rodent brain (for review see (46)). Ependymomas and choroid plexus papillomas can be induced by SV40 and BK virus in hamster brain. JC virus, the pathogenetic agent in progressive multifocal leucoencephalopathy (PML) in humans, causes ependymomas, pineocytomas, extracranial neuroblastomas and medulloblastomas after inoculation into hamster brain. In addition, glioblastomas and astrocytomas developed after intracerebral injection of JC virus into owl and squirrel monkeys (46). All three viruses are capable of immortalizing human cells *in vitro* mainly due to the action of proteins encoded by the viral early region, i.e. large and small TAg (19, 40). Recently, authentic SV40 genomes have been reported in human ependymomas, choroid plexus papillomas, osteosarcomas, mesotheliomas and papillary thyroid carcinomas (8, 11, 29, 30, 37, 43). Complex formation between the SV40 large TAg and the cellular tumor suppressors p53, retinoblastoma protein (RB), p107 and p130 has been demonstrated in human mesotheliomas (9, 13). JC virus sequences were detected in human colorectal cancers and shown to be expressed in human medulloblastomas and in a single immunocompetent patient with oligoastrocytoma (27, 28, 38) and indirect evidence implicates JC virus in the “rogue cell” type chromosomal damage (35). BK virus sequences have also been reported to be present in a large proportion of normal human tissues and tumors of different histogenetic origins and interaction between the BK virus large T antigen and cellular p53 has been detected in neuroblastomas by immunoprecipitation analysis (14, 20).

The transforming activity of polyomavirus large TAg largely relies on its ability to complex and inactivate p53 and proteins of the RB family (19). To further elucidate the incidence of polyomaviruses in human brain tumors we have therefore focussed on tumors that rarely contain alterations in the p53 tumor suppressor pathway, namely medulloblastomas, meningiomas and ependymomas (25). Hepatoblastomas were included as extracranial malignancies that can be induced by liver directed large TAg expression in transgenic mice (7).

Material and Methods

Tumor and blood samples and DNA extraction. A total of 116 medulloblastomas (including 7 cell lines), 131 meningiomas, 25 ependymomas, 2 subependymomas, 60 hepatoblastomas (including 4 cell lines) and 31 brain samples from schizophrenic patients were retrieved from the files of the Department of Neuropathology and the Department of Psychiatry, University of Bonn Medical Center and the Department of Neuropathology, Heinrich-Heine-University Düsseldorf. All tumors were classified according to the WHO guidelines (24). The tumor fragments used for DNA extraction were carefully checked by frozen section to rule out contamination by necrotic or normal tissue. High molecular weight DNA was extracted from snap-frozen biopsies or blood samples by standard proteinase K digestion and phenol chloroform extraction. DNA from 18 paraffin-embedded medulloblastoma samples was extracted using the Qiamp Tissue kit (Qiagen). The integrity of the DNA samples was validated with PCR amplifications of genomic sequences.

PCR analysis and sequencing. PCR reactions were carried out in a final volume of 10 μl containing 100 ng of DNA, 10 pmol of each primer, 10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ (1.0 mM $MgCl_2$ for primers LA1/LA2 and JV3/JV4) and 200 mM of each nucleotide in a thermocycler (Biometra). The reaction products were diluted with 10 μl deionized formamide (Ambion) and 2 μl analysed on a 6% denaturing acrylamide gel with a semi-automated DNA sequencer (LI-COR, Model L-4200 S-1) and the 1D-Scan software (MWG-Biotech, Germany). All samples were screened with two sets of primers previously used by Bergsagel *et al*. (3). Primers TAGGTGCCAACCTATGGAACAGA, (PYV.for) and GGAAAGTCTTTAGGGTCTTCTACC, (PYV.rev) amplify sequences spanning the RB pocket domain of large TAg common to the polyomaviruses SV40, JC and BK with a size of 172 bp for SV40, 178 bp for JC virus and 181 bp for BK virus. Primers TGAGGCTACTGCTGACTCTCAACA, (SV.for3) and GCATGACTCAAAAAACTTAGCAATTCTG, (SV.rev) were used to amplify a SV40 specific 105 bp fragment that partially overlapped the TAg fragment amplified with primers PYV.for and PYV.rev. For both primer sets, initial denaturation at 94° for 3 min was followed by 40 cycles of 94° for 40s, 53° for 60s and 72° for 60s. Final extension was carried out for 10 min at 72°. As a positive control for amplification of the SV40 Tag, DNA of the plasmid pZIPneoSV(TAg) (gift of Dr. P. Jat, London, Great Britain) containing the viral early region was used. As a positive control for amplification of the JC virus TAg, DNA was extracted from the snap-frozen brain sample of an AIDS patient with PML (kindly provided by Drs. A. Aguzzi and T. Voigtländer, Zürich, Switzerland). All amplifications were carried out in duplicate and positive as well as negative controls were always included. Positive results were reproduced at least five times. SV40 positive samples were further analysed for the presence of VP-1 late gene sequences using primers GGGTGTTGGGCCCTTGTGCAAAGC, (LA1) and CATGTCTGGATCCCCAGGAAGCTC, (LA2). Amplification results in a PCR product of 294bp (29). Initial denaturation at 94° for 3 min was followed by 40 cycles at 94° for 40s, 63° for 60s and 72° for 60s. Final extension was carried out for 10 min at 72°. As a positive control for amplification of the SV40 VP-1 late gene, DNA of the plasmid pBSV776.1 (gift of Dr. K.-H. Scheidtmann, Bonn, Germany) containing the entire viral genome was used. JC positive samples were confirmed with primers TTCTTTCCTTCTTACTGATTTA, (JV3) and TATGACTTTGCTTTTGCTTTTA, (JV4). These primers amplify a 602 bp fragment spanning a domain of the JC genome in which both early and late transcription units terminate (22). Initial denaturation at 94° for 3 min was followed by 40 cycles at 94° for 60s, 50° for 30s and 72° for 60s. Final extension was carried out for 10 min at 72°. For sequencing, primary PCR products with primers PYV.for/PYV.rev or SV.for3/SV.rev were reamplified with another 40 cycles using the same primers, cloned using the TOPO TA cloning kit (Invitrogen) and sequenced using M13 standard primers. Cycle sequencing was carried out using a fluorescent dideoxy terminator kit (ABI) and products were analysed on an Applied Biosystems model 373A DNA sequencer.

A limited series of 7 ependymomas and 7 medulloblastomas was also analysed using a sensitive blotting assay as reported by Huang *et al*. (23). Selected cases have also been amplified with higher amounts of tem-

Sample no.	Tumor type	Sex	Year of birth/Age at diagnosis	SV40 positive(PCR)	JC virus positive(PCR)	Immunostaining
D40	Medulloblastoma WHO grade IV	M	1967/7	+	-	negative
D212II	Medulloblastoma WHO grade IV	M	1982/14	+	-	n.d.
276	Anaplastic Ependymoma WHO grade III	M	1960/25	+	-	n.d.
290	Subependymoma WHO grade I	M	1914/76	+	-	n.d.
596	Anaplastic Meningioma WHO grade III	F	1943/49	+	-	negative
2206	Meningioma WHO grade I	F	1948/45	-	+	negative

Table 1. Detection of polyomavirus sequences in human brain tumors.

plate genomic DNA as well as higher cycle numbers (up to 45 cycles) without additional positive results (data not shown).

Immunohistochemical analysis of tumor biopsies. For immunohistochemical studies of tumor samples, monoclonal antibody 101 against SV40 large T antigen (gift of Dr. K.-H. Scheidtmann) was used. The epitope of antibody 101 (1:100) is located in the C-terminal half of the protein (amino acids 522-610). Neonatal rat brain after inoculation with the large TAg expressing tumor cell line TZ102-1 was used as a positive control for antibody 101 (48). The polyclonal antiserum NCL-JC (Novocastra; Clone 5.12.2; 1:10) recognizes the major capsid protein VP-1 of JC virus. Brain sections of an AIDS patient with PML served as a positive control for antibody NCL-JC. Tumor samples and positive control samples were fixed for 12-24 h in paraformaldehyde. To enhance the immunoreactivity in paraffin embedded brain tissue, sections were heated in a microwave oven in 0.01 M citrate buffer, pH 6.0. Sections were preincubated with 10% FCS in PBS, 5% non fat dry milk (Biorad) and with normal goat serum (DAKO; prior to the addition of polyclonal antibodies) or normal horse serum (DAKO; prior to the addition of monoclonal antibodies) to block non-specific binding sites. This was followed by an overnight incubation with the primary antibody at room temperature. Staining was visualized using the ABC method and a Vectastain kit (Vector Laboratories). Counterstaining was carried out with hematoxylin.

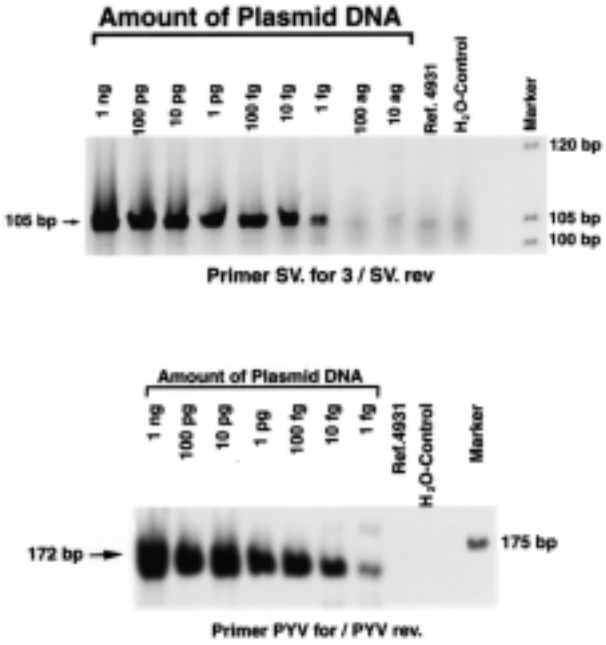

Figure 1. Sensitivity of the PCR-assay used to identify polyomavirus sequences in human brain tumors. Different amounts of plasmid pZIPneoSV(TAg) (containing the SV40 viral early region) were mixed with 200 ng of DNA prepared from peripheral blood lymphocytes negative for polyomavirus sequences (Ref 4931). PCR was performed for 40 cycles with primers SV.for3/SV.rev or primers PYV.for/PYV.rev. and the PCR products were analysed with a semi-automated DNA sequencer (LI-COR). Both primer sets detected approximately 1 fg plasmid-DNA diluted in 200 ng PBL-DNA. This indicates that PCR was capable of amplifying less than 1 viral genome per 250 cells.

Results

Sensitivity of the PCR assay. All DNA samples were screened with the SV40 specific primers SV.for3/SV.rev and primers PYV.for/PYV.rev that amplify sequences spanning the RB pocket domain of T-antigen common to SV40, JC and BK virus (3). To determine the detection limit of the PCR assay, we serially diluted the plasmid pZIPneoSV(TAg) (encoding the SV40 viral early region) into 200 ng of DNA prepared from peripheral blood lymphocytes. PCR was then performed with 40 cycles of amplification. Both primer sets detected approximately 1 fg plasmid-DNA diluted in 200 ng PBL-DNA. Assuming that a diploid cell contains approximately 6 pg of DNA, 200 ng of DNA roughly correspond to 33,000 cells. This indicates that the PCR assay was capable of detecting less than 1 viral genome per 250 cells (Figure 1).

SV40 and JC virus sequences in human medulloblastomas, meningiomas and ependymomas. Initial screening detected SV40 sequences in 2/116 medul-

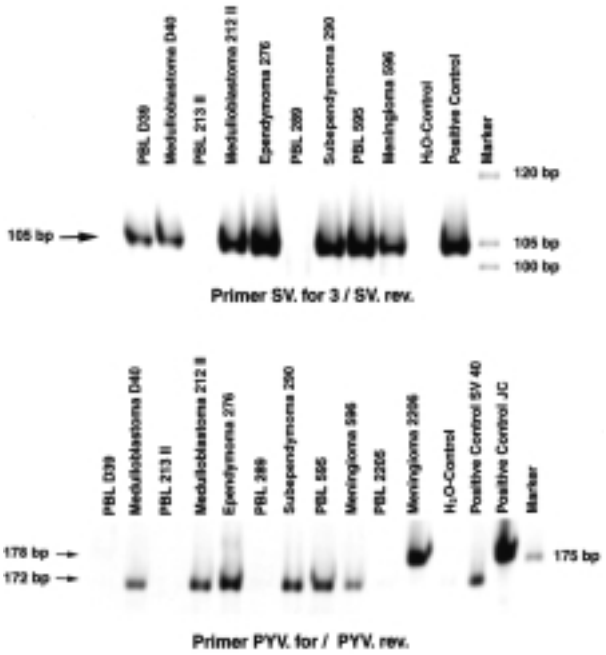

Figure 2. Detection of SV40 and JC virus sequences in human medulloblastomas, meningiomas, and ependymomas. Primers SV.for3/SV.rev., SV40 virus sequences were detected in medulloblastomas D40 and D212II, in meningioma 596, in ependymoma 276 and in the subependymoma 290. The size of the PCR product was 105 bp (3). Note that the corresponding peripheral blood lymphocyte (PBL) sample D39 of one medulloblastoma patient (D40) and PBL 595 of the meningioma patient (596) were also positive for SV40 T-antigen sequences. A PBL sample was not available from ependymoma patient 276. Primers PYV.for/PYV.rev amplify sequences spanning the RB pocket domain of TAg common to SV40, JC and BK virus (3). Screening the panel of tumor samples with this primer set confirmed the results with primers SV.for3/SV.rev. Only amplification of PBL DNA D39 failed to elicit a PCR fragment of 172 bp possibly indicating a partial deletion of the T-antigen region in the lymphocytes of this medulloblastoma patient. In addition, these primers detected JC virus TAg sequences in the meningioma 2206 with a product size of 178 bp. The corresponding PBL DNA 2205 was negative.

loblastomas (medulloblastomas D40 and D212II), in 1/131 meningiomas (meningioma 596), in 1/25 ependymomas (ependymoma 276) and in 1/2 subependymomas (subependymoma 290; Table 1; Figure 2). Interestingly, two patients with tumors positive for SV40 sequences (medulloblastoma D40 and meningioma 596) also showed virus DNA in their PBL samples. All specimens positive for SV40 T-antigen sequences were further analysed for the presence of VP-1 late gene sequences. Primers LA1/LA2 detect a segment of the VP-1 gene encoding the C-terminus of the major capsid protein (29). The specific 294 bp fragment of the VP-1 gene could be amplified only from DNA of ependymoma 276 (not shown). In addition, a single meningioma (meningioma 2206) displayed JC virus large TAg sequences (Table 1; Figure 2). The PBL sample 2205 of this patient was negative. The presence of JC virus sequences in meningioma 2206 was confirmed with primers JV3 /JV4 (22) that amplify a 602 bp fragment spanning the part of the JC genome in which both early and late transcription units terminate (not shown). Sequencing of the PYV.for/PYV.rev or SV.for3/SV.rev PCR products verified the presence of SV40 large TAg or JC large TAg (meningioma 2206) sequences in all cases except D40 where sufficient DNA was not available. In contrast, 60 hepatoblastoma samples and 31 brain samples from schizophrenic patients were consistently negative. We could not observe preferential association of SV40 sequences with a specific tumor type. Moreover, BK virus sequences were not detectable in any of our samples.

Immunohistochemistry. Immunohistochemical analysis of medulloblastoma D40 and meningioma 596 with monoclonal antibody 101 against large TAg did not show expression in the tumor cells or in non-neoplastic cell types of these specimens (Table 1; Figure 3A). In the JC positive meningioma 2206, immunoreactivity for the viral late gene product (VP-1) was absent (Table 1; Figure 3B). Positive control samples always elicited a strong and reproducible immunohistochemical reaction (Figure 3A and B, inserts).

Discussion

Poliovirus vaccine used in the United States between 1955-63 has been extensively contaminated with SV40, a macaque polyomavirus that is tumorigenic in rodents and able to immortalize human cells *in vitro* (6, 10, 40, 46). Recently, authentic SV40 sequences have been described in human ependymomas, choroid plexus papillomas, osteosarcomas and papillary thyroid carcinomas and complex formation between the SV40 large T-antigen and p53 as well as RB has been shown in human mesotheliomas (9, 13, 29, 30, 37, 43). In papillary thyroid carcinomas and osteosarcomas the SV40 DNA has been shown to be integrated within chromosomal DNA (33, 37). These findings have raised the possibility that exposure to SV40 contaminated poliovirus vaccine may be associated with increased rates of brain tumors, osteosarcomas or mesotheliomas. Although recent epidemiological studies argue against this hypothesis, the significance of polyomavirus sequences in human tumors is still a matter of intense debate (36, 42).

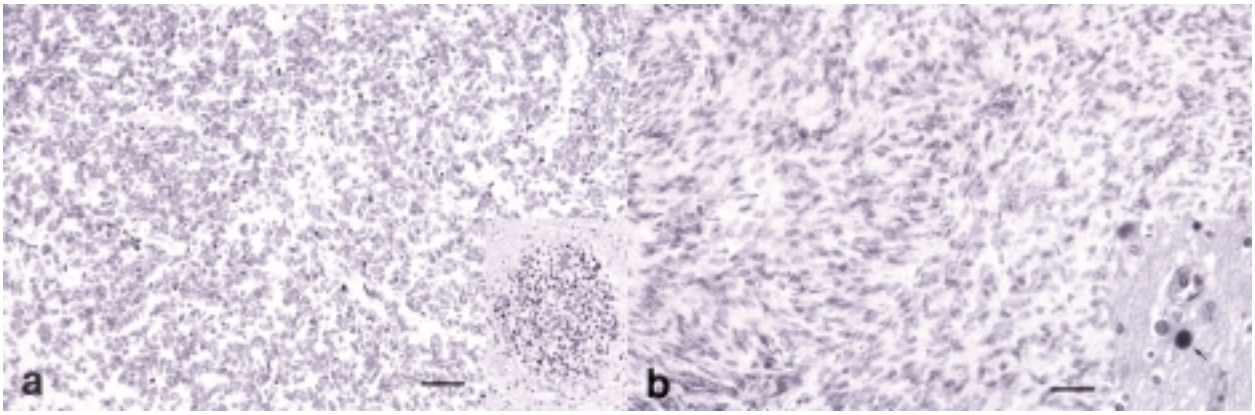

Figure 3. Immunohistochemical analysis of brain tumor biopsies. **A.** Staining of medulloblastoma D40 with antibody 101 against large T-antigen. The tumor is completely negative indicating that large TAg is not expressed in the tumor cells. Meningioma 596 likewise did not show positive staining (not shown). From medulloblastoma D212II, ependymoma 276 and subependymoma 290 tumor sections were not available for staining. The insert shows neonatal rat brain after inoculation with the large TAg expressing tumor cell line TZ102-1 as a positive control for antibody 101 (48). Scale bar equals 50 μm. **B.** Staining of meningioma 2206 with antibody NCL-JC against the major capsid protein VP-1 of JC virus. No specific immunoreactivity was observed. The insert shows positive staining of JC virus infected oligodendrocytes in the brain of an AIDS patient with PML (arrow). Scale bar equals 50 μm.

In the present study we identified simian virus 40 (SV40)-like sequences in 2/116 medulloblastomas, in 1/131 meningiomas, in 1/25 ependymomas and in 1/2 subependymomas. One of the meningioma samples showed JC virus sequences. Overall our data suggest that the incidence of SV40, JC and BK virus sequences in human brain tumors is considerable lower than reported by other authors. Using the primers PYV.for/PYV.rev Bergsagel *et al*. (3) described SV40 T-antigen sequences in 10/11 ependymomas but could not detect SV40 sequences in 100 control peripheral blood lymphocyte (PBL) samples from children. Using a polyclonal antibody against SV40 large T-antigen, these authors also reported expression of T-antigen in 3 ependymoma biopsies. With the identical primers, Martini *et al*. (32) reported SV40 T-antigen sequences in 73% of ependymomas, 83% of choroid plexus papillomas, 47% of astrocytomas, 33% of glioblastomas and 14% of meningiomas. In a recent paper Huang *et al*. (23) using SV40 specific primers described the presence of T-antigen DNA sequences in 56% of ependymomas, 38% of choroid plexus papillomas, 29% of medulloblastomas, 44% of astrocytomas and 30% of glioblastomas. However, although largely similar in the frequencies of SV40 DNA sequences these two latter studies show astonishing differences for the detection of BK virus sequences in brain tumor samples. Whereas an earlier report demonstrated the presence of BK virus sequences in the vast majority of tumor samples investigated by Martini *et al*. (14, 32), Huang *et al*. (23) detected BK virus DNA sequences only with a very low overall frequency of 3%. Earlier studies using classical Southern-blot hybridization have also suggested the presence of SV40 and BK virus DNA in human meningiomas (15, 26). Other investigators were unable to demonstrate polyomavirus DNA sequences in human brain tumors (1, 12, 22). The explanation for this substantial disagreement between our data and, most strikingly, the data by Martini *et al*. (32), Huang *et al*. (23) and Krynska *et al*. (27) is presently unclear but several explanations need to be discussed.

For detection of polyomavirus DNA sequences most studies use PCR amplification of viral sequences with subsequent Southern-blotting. We have developed an alternative assay with fluorescence-labelled primers and detection of the PCR products with an automated DNA sequencer. The main advantage of this approach is that it allows high-throughput screening of tissue samples which was necessary for our large tumor panel. Futhermore, using the primers PYV.for/PYV.rev that amplify sequences spanning the RB pocket domain of large TAg common to the polyomaviruses SV40, JC and BK, the high resolution of sequencing gels allows direct identification of the target virus by the size of the resulting PCR product. This avoids possible cross hybridisation between closely related virus sequences in the Southern-blotting procedure. Finally, we have shown that the PCR assay was capable of detecting less than 1 viral genome per 250 cells, which is in the range of other studies that have determined the detection limit for PCR with subsequent Southern-blotting (3, 16, 33, 44). Some laboratories have reported on very low amounts of

polyomavirus sequences in PBL samples as well as brain tissue from healthy or at least immunocompetent control individuals while others were not able to detect polyomavirus sequences in comparable samples (2, 3, 5, 14, 16, 17, 32, 44, 45, 47, 49). We have also screened a large sample of PBL samples from adult German volunteers and did not detect polyomavirus sequences (S. Weggen, unpublished data). Likewise, 31 brain samples from schizophrenic patients tested negative in our study. Although our PCR assay should have been able to detect minute amounts of polyomavirus T-antigen sequences these contradictory findings may be the result of an even higher sensitivity of the detection assays in other studies (2, 23). However, in these studies the detection limit was determined with experimental conditions that do not reflect the detection of viral sequences with a background of human genomic DNA (2, 23). In order to compare the sensitivity of our detection assay with the Southern blotting-based strategy used in ref. 23, Huang *et al*. have analysed 14 samples from our series (7 medulloblastomas and 7 ependymomas) with their blotting protocol and two different sets of SV40 specific PCR primers. These experiments have yielded results identical to our findings. Those two specimens which showed SV40 TAg sequences in our series (medulloblastoma D212II and ependymoma 276) were also positive with the alternative approach. The remaining twelve tumor DNAs were consistently negative in both laboratories. This observation strongly indicates that a variable detection sensitivity cannot account for the significant differences in the rate of polyomavirus DNA-containing tumors in the studies from Bonn and from Lyon. In this context it should be stressed that the presence of polyomavirus sequences in PBLs could constitute a serious pitfall for the interpretation of virus sequences in tumor samples. Since tumors are always contaminated by circulating PBLs positive PCR results in tumors may originate from these peripheral cells and not from the tumor cells. Therefore it appears mandatory to prove the presence of viral genomic sequences or the expression of viral genes in the tumor cells by in situ hybridisation or immunohistochemistry. This has not been achieved in most of the studies reporting a high incidence of polyomavirus DNA sequences in brain tumors (23, 32).

Lednicky *et al*. (30) have suggested that the identification of SV40 sequences may be seriously impaired when DNA from paraffin-embedded tissue is used for PCR. However in our analysis, the DNA of only 18/131 medulloblastomas was extracted from paraffin-embedded tissue; all other DNA samples were obtained from snap-frozen biopsies. Therefore, this should not account for the low frequency of polyomavirus sequences in our samples. The recovery of non-integrated episomal virus DNA sequences may also depend on the method used for DNA extraction. However, the significance of episomal DNA in human tumors is still unclear.

Geographical differences that in the case of SV40 could be related to the use of contaminated poliovaccines have also been proposed as possible cause for the divergent reports of polyomavirus DNA sequences in human tumors (11). Recently, Linnainmaa *et al*. (31) failed to detect SV40 DNA sequences in mesotheliomas from Finland but the same authors were able to confirm the presence of SV40 sequences in mesotheliomas from the United States in a multi-institutional study (43). In this regard it may be of interest that both SV40 positive ependymomas were from U.S. patients whereas all other tumor samples including ependymomas were from patients in central Europe. However, the small number of positive ependymomas in our study precludes any statistical analysis. Furthermore, this is still in contrast to the positive findings in Italian and Swiss ependymomas (23, 32).

Two recent studies investigated the presence of polyomavirus sequences in a considerable collection of medulloblastomas (23, 27). In agreement with Huang *et al* (23), we were not able to detect JC virus sequences in tumors from European patients. In contrast, Krynska *et al* (27) found JC virus sequences in 11 of 23 medulloblastomas in a recent study on U.S. patients. Medulloblastoma-like tumors develop at a high incidence following intracerebral inoculation of JC virus into newborn hamsters (46). This animal model and the ability of SV40 large TAg to induce various brain tumors with features of primitive neuroectodermal tumors in transgenic mice made medulloblastoma a special candidate for an involvement of polyomaviruses in its pathogenesis (21). Hepatocellular carcinomas and concomitant hepatoblastomas can also be induced in transgenic mice by liver targeted expression of SV40 large TAg. There is strong evidence that hepatitis viruses participate in the development of human hepatocellular carcinomas but a viral etiology for hepatoblastomas has not been explored (4, 34). The failure to amplify polyomavirus sequences in our large panel of hepatoblastomas appears to rule out a pathogenic role for this group of DNA viruses in these liver malignancies.

The detection, although at low frequency, of SV40 and JC virus sequences in brain tumor samples cannot exclude these viruses as an initiative agent in the setting of a hit-and-run mechanism. In transgenic mice with

inducible large TAg expression, it was indeed shown that tumors eventually lose their dependence on TAg for maintenance of the transformed state (18). Even more striking, a SV40 large TAg induced rat brain tumor cell line displayed enhanced malignancy after reproducible loss of the large TAg coding region and concomitant p53 mutation (39). However, the low incidence of virus sequences and the absence of T-antigen protein expression argue against a key role of these viruses in the development of human medulloblastomas, meningiomas and ependymomas.

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