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Lack of association of herpesviruses with brain tumors

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Gliomas are the most frequent primary brain tumors in humans. Many studies have been carried out on their etiology; however, the only confirmed risk factors are hereditary predisposing conditions and high dose of ionizing radiation. Recently, human cytomegalovirus (HCMV) gene products and nucleic acids were reported to be present in all of 27 glioma samples investigated in contrast to other brain tissues, and it was hypothesized that HCMV might play a role in glioma pathogenesis. To evaluate these findings, samples of 40 gliomas, 31 meningiomas, and 6 acoustic neurinomas (ACNs) were analyzed for the presence of HCMV macromolecules using polymerase chain reaction (PCR) and immunohistochemistry. Additionally, corresponding blood samples from 72 patients were analyzed for the presence of HCMV DNA to check for a possible contamination of tumor tissues with HCMV-infected blood cells. No HCMV DNA sequences were found, neither in brain tumor tissues nor in corresponding blood samples. Immunohistochemistry did not detect HCMV-specific proteins. Addressing a possible role of other herpesviruses as has been suggested in seroepidemiological studies, seroprevalences of antibodies to HCMV, herpes simplex virus (HSV), Epstein-Barr virus (EBV), and varicella-zoster virus (VZV) were determined by enzyme-linked immunosorbent assay (ELISA). Serological analyses of brain tumor patients showed no significant differences in the prevalences of antibodies to HCMV, HSV, EBV, or VZV compared to the general population. Thus, the data of the present study do not support the hypothesis of an association of herpesviruses with the development of primary brain tumors. *Journal of NeuroVirology* (2006) **12,** 90–99.

Keywords: Epstein-Barr virus; herpes simplex virus; human cytomegalovirus; varicella-zoster virus

Introduction

In Germany, primary brain tumors are among the 20 most frequent causes of cancer deaths (Becker, 1998). Etiologically, the only confirmed risk factors are hereditary predisposing conditions (e.g., Li-Fraumeni syndrome, neurofibromatosis, and tuberous sclerosis) and high dose of ionizing radiation. However, these factors only account for less than 5% of all incident primary brain tumors (Preston-Martin and Mack, 1996; Wrensch *et al*, 2002). Many other putative risk factors for brain tumor pathogenesis have been investigated. These include occupational exposures and electromagnetic fields as well as medical risk factors such as head injuries, allergies, and atopic and infectious diseases (Menegoz *et al*, 2002; Preston-Martin *et al*, 1998; Schlehofer *et al*, 1992, 1999; Bondy and Wrensch, 1996; Inskip *et al*, 1995; Wrensch *et al*, 2002; Preston-Martin and Mack, 1996). A possible role of viral infections, such as simian virus 40, JC virus, BK virus, and members

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of the family of herpesviruses, has been controversially discussed during the last decades (Smith *et al*, 1974; Pagano *et al*, 2004). Numerous studies have addressed the occurrence of viral products in tumor tissues (Inskip *et al*, 1995; Wohlrabe *et al*, 1984) as well as seroprevalences of antiviral antibodies (Hadfield *et al*, 1984; Wrensch *et al*, 1997, 2001; Rollison *et al*, 2003).

In 2002, it was hypothesized that human cytomegalovirus (HCMV) might be involved in the development or progression of gliomas (Cobbs *et al*, 2002). In all 27 glioma biopsies examined in their study, expression of multiple HCMV gene products was detected in contrast to samples from other primary brain tumors, several nontumor brain diseases, and normal brain tissue.

HCMV is a ubiquitous β-herpesvirus (human herpesvirus-5) that is most feared in immunocompromised persons, where it can cause severe and fatal diseases such as HCMV encephalitis and graft rejection (Britt and Alford, 1996). Furthermore, it is the most frequent cause of congenital malformations (Landolfo *et al*, 2003). Infants with congenital HCMV infection are more prone to disorders involving the perceptual organs and the nervous system (Ho, 1990). It is well known that HCMV is trophic for glial cells (Fritschy *et al*, 1996), and it has been shown that HCMV can be activated in astrocytic cells by inflammatory processes (Wolff *et al*, 1994). Furthermore, HCMV can induce malignant transformation *in vitro* (Doniger *et al*, 1999), possibly by its ability to repress cell growth arrest and p53-mediated apoptosis response (Castillo *et al*, 2000; Castillo and Kowalik, 2002; Lokensgard *et al*, 1999).

Studies concerning putative viral risk factors for brain tumor development have addressed the role of previous herpesvirus infections by evaluating the prevalences of antibodies to HCMV, varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and herpes simplex virus (HSV) in brain tumor patients. An inverse association between glioblastoma cases and the occurrence of immunoglobulin (Ig) G antibodies to VZV and EBV has been reported. In contrast, glioblastoma cases were more likely to have antibodies to HSV and HCMV than controls (Wrensch *et al*, 1997, 2001). Furthermore, it has been reported that patients with cerebral tumors were more likely to have an acute herpesvirus infection; however, the mean geometric antibody titers against herpesvirus in brain tumor patients did not differ from healthy controls (Wohlrabe *et al*, 1984). In another study, glioblastoma cases and pituitary adenoma patients were found to have higher titers of HSV serum antibodies than patients with astrocytoma, medulloblastoma, meningioma, or metastatic tumors (Hadfield *et al*, 1984). All these studies suggested that several types of herpesviruses might be involved in glioma pathogenesis.

The aim of the present study was (i) to assess the prevalence of HCMV gene products and nucleic acids in primary human brain tumors, and (ii) to assess the prevalence of anti-HCMV, anti-VZV, anti-EBV, and anti-HSV IgM and IgG antibodies in sera of brain tumor patients to estimate the role of previous herpesvirus infections in tumor development.

Results

Sample collection

In the present study, 77 brain tumor patients (40 gliomas, 31 meningiomas, 6 acoustic neurinomas) were included, with no age restriction for participation. Corresponding blood samples were provided from 72 of the patients (93.5%; 5 glioma patients did not provide a blood sample). Furthermore, from 73 of these tumor tissues, paraffin-embedded material could be obtained for immunohistochemical analyses (94.8%; 38 gliomas, 29 meningiomas, 6 acoustic neurinomas).

Overall, patients had a median age of 53 years with a range from 9 to 83 years. The median age of meningioma and glioma patients was similar with 54.5 and 53 years, respectively; acoustic neurinoma (ACN) patients were younger with a median of 42 years (Table 1). Fifty-five percent of glioma patients and 67% of ACN patients were male, whereas the majority of meningioma patients was female (84%).

The histologies and the particular analyses performed with the samples are shown in Table 2.

Polymerase chain reaction (PCR) analyses

To assess the presence of HCMV DNA in primary brain tumors, nested PCR analyses were performed with various primers amplifying sequences of different HCMV-specific genes (immediate early [IE] 1, glycoproteinB). PCR of corresponding blood samples of the patients performed as a specificity control for tumor tissues did not amplify any HCMV DNA sequences. Similarly, HCMV DNA was not detected, neither in the brain tumor samples nor in short-term cultures derived from one glioma, two meningiomas, and one ACN with any of the different PCR protocols used (Figure 1; Table 2).

Table 1 Distribution of age and gender in 77 brain tumor patients

		Age		*Gender*			
		Median age	*Age range*	*Male*		*Female*	
	N	*(years)*	*(years)*	n	*(%)*	n	*(%)*
Glioma patients	40	54.5	9–80	22	(55.0)	18	(45.0)
Meningioma patients	31	53.0	32–83	5	(16.1)	26	(83.9)
ACN* patients	6	42.0	34–66	4	(66.7)	2	(33.3)

*ACN, acoustic neurinoma.

Table 2 Lack of detection of HCMV molecules in brain tumor tissue and corresponding blood samples using different methods (number of positive probes out of the number of samples tested)

		PCR tumor tissue (n = 77)		*IHC tumor tissue (n = 73)[a]*			*PCR blood samples (n = 72)[b]*	
		Primers specific for		*Monoclonal antibodies to*			*Primers specific for*	
Tumor type	N (n = 77)	*gB*	*IE1*	*pp65*	*IE*	*EA*	*gB*	*IE1*
Glioblastoma multiforme IV	23	0/23	0/23	0/22	0/22	0/22	0/22	0/22
Gliosarcoma IV	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Anaplastic oligodendroglioma III	4	0/4	0/4	0/4	0/4	0/4	0/2	0/2
Astrocytoma II/III	3	0/3	0/3	0/2	0/2	0/2	0/2	0/2
Diffuse astrocytoma II	3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Oligodendroglioma II	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Glioma (not further specified)	5	0/5	0/5	0/5	0/5	0/5	0/4	0/4
Transitional meningioma I	10	0/10	0/10	0/9	0/9	0/9	0/10	0/10
Meningothelial meningioma I	5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Microcystic meningioma I	2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Fibrous meningioma I	2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Psammomatous meningioma I	1	0/1	0/1	0/0	0/0	0/0	0/1	0/1
Secretory meningioma I	2	0/2	0/2	0/2	0/2	0/2	0/2	0/2
Meningioma I	6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
Atypical meningioma II	3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Acoustic neurinoma I	5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Acoustic neurinoma II	1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Tumors in total	77	0/77	0/77	0/73	0/73	0/73	0/72	0/72

Abbreviations: gB, glycoproteinB; IE, immediate early; pp65, phosphoprotein 65; EA, early antigen; IHC, immunohistochemistry.
[a]No paraffin slides available of two glioma and two meningioma patients.
[b]No blood samples available for five glioma patients.

To exclude a selection bias in the present study population, additional PCR analyses were performed with glioma short-term cultures derived from patients distinct from the study participants. Also in these samples (three gliomas World Health Organization [WHO] grade III, one glioblastoma WHO grade IV), HCMV DNA could not be found.

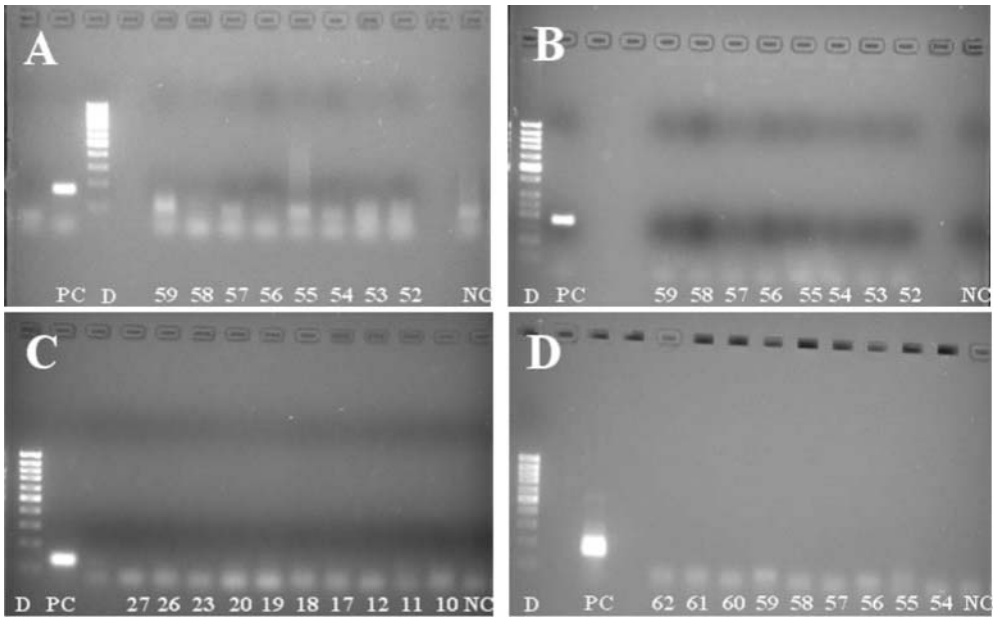

Figure 1 Representative examples of nested PCR results for HCMV detection in brain tumor tissue (**A, B**) and corresponding blood samples (**C, D**). PC: positive control (cell-lysate of HCMV-infected cells); NC: negative control (double-distilled water); D: size marker (DNA ladder). Samples: glioma with unknown grading (10, 20); glioma WHO II (26, 54, 56); glioma WHO III (60); glioblastoma (52, 53, 55, 58, 61); meningioma WHO I (11, 12, 17, 18, 19, 23, 27, 57, 62); ACN (59). (**A**) and (**C**) show an agarose gel after nested PCR amplifying the HCMV-specific gB (UL55) gene. (**B**) and (**D**) show an agarose gel after nested PCR amplifying the HCMV-specific IE1 gene. The positive controls show a band at the expected target size, whereas no amplified fragment was found in brain tumor tissues and blood samples.

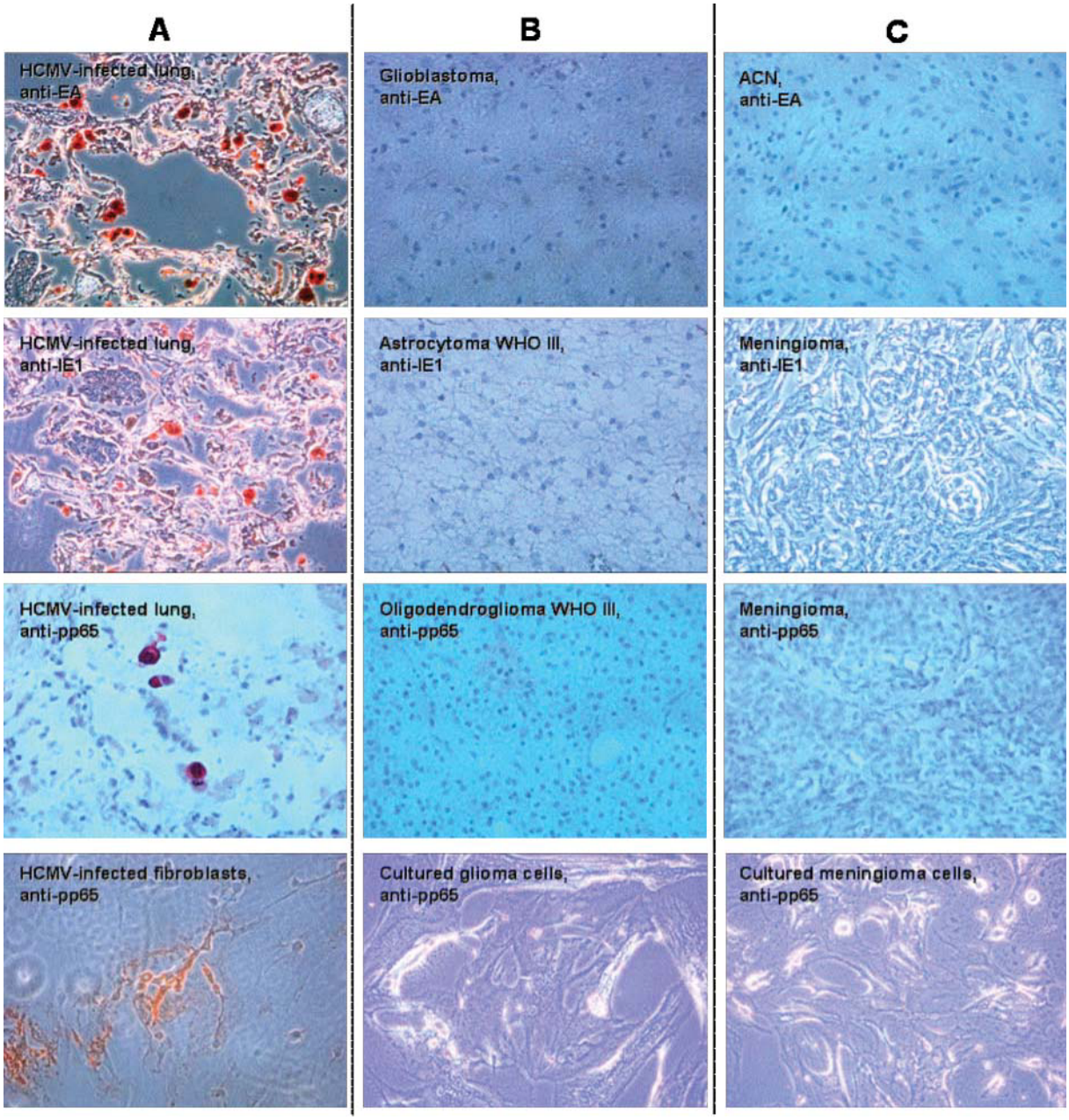

Figure 2 Immunohistochemistry for HCMV detection in brain tumor sections. Tissue types and anti-HCMV antibodies used (pp65, IE1, EA) are indicated in the respective micrograph. Typical enlarged cells can be seen in the positive controls (HCMV-infected lung tissue and HCMV-infected fibroblasts, respectively) with HCMV-immunoreactivity (*red staining*) in both cytoplasm and nucleus (column **A**). The lack of immunoreactivity in brain tumor tissue sections (first, second, and third rows) and corresponding short-term cultures (last row) is visible in columns **B** and **C**.

Immunohistochemistry

The presence of HCMV-specific proteins in 73 brain tumor samples was investigated by immunohistochemistry using three different monoclonal antibodies (anti-IE, anti-pp65, and anti–early antigen). In addition to the brain tumor samples, short-term cultures derived therefrom (two meningiomas, two gliomas) were tested for the presence of the HCMV-specific pp65 protein. No immunoreactivity was detected in the 73 primary brain tumors nor in the four short-term cultures tested with any of the monoclonal antibodies used. In contrast, positive controls (HCMV-positive lung tissue and HCMV-infected fibroblasts, respectively) showed clear intracytoplasmatic and intranuclear staining (Figure 2).

Table 3 Overall prevalences and 95% confidence intervals (CI) of subjects positive for IgG antibodies to HCMV, HSV, EBV, and VZV

	IgG positive patients n	*Brain tumor patients (n = 72)**		*Published prevalences*	
		Prevalence (%)	*(95% CI)*	*Prevalence (%)*	*Reference*
HCMV	46	63.9	(52, 75)	40—80	RKI, 2000a
				42	Krech, 1973 (20–40-year-old)
HSV	62	86.1	(76, 93)	83	Hellenbrand *et al*, 2005
				—	—
EBV	64	88.9	(79, 95)	>90	Cohen, 2000
				—	—
VZV	66	91.7	(83, 97)	>90	RKI, 2000b
				>90	Wutzler *et al*, 2001

*No blood samples available for five glioma patients.
RKI, Robert-Koch Institute; IgG, immunoglobulin G; HCMV, human cytomegalovirus; HSV, herpes simplex virus; EBV, Epstein-Barr virus; VZV, varicella-zoster virus.

Serological analyses

To investigate the role of previous herpesvirus infections in brain tumor etiology, seroprevalences of IgM and IgG antibodies to four herpesviruses (HCMV, HSV, EBV, and VZV) could be evaluated in 72 of the 77 patients. Five glioma patients did not provide a blood sample; the distribution of age and gender in glioma patients providing sera did not differ from that of all glioma patients included in the study as given in Table 1 (data not shown).

IgM antibodies against the four herpesviruses investigated (HCMV, HSV, EBV, and VZV) were not found in any of the serum samples.

Analyses of IgG seroprevalences and the corresponding exact 95% confidence intervals (CIs) revealed overall seroprevalences of IgG antibodies to HCMV of 64% (95% CI 51%–75%) and of 86% (95% CI 76%–93%) for HSV antibodies. The study participants showed a seroprevalence of IgG antibodies to EBV of 89% (95% CI 79%–95%) and a prevalence of anti-VZV IgG antibodies of 92% (95% CI 83%–97%) (Table 3).

Analyses stratified by tumor type showed no substantial differences in the seroprevalences (Table 4). In analyses stratified by 20-year age groups, prevalences were increasing with increasing age for IgG antibodies to HCMV and HSV, and decreasing for antibodies to EBV (Table 4). The highest prevalence of anti-VZV antibodies was observed in the age group 40–59 years.

As shown in Table 3, the prevalences of antibodies to HCMV, HSV, EBV, or VZV in brain tumor patients showed no significant differences compared to

Table 4 Seroprevalences (SP) and corresponding exact 95% confidence intervals (CI) of immunoglobulin G (IgG) antibodies to HCMV, HSV, EBV, and VZV in 72 brain tumor patients, stratified by tumor type and 20-year age groups

		Immunoglobulin G antibody–positive patients with								
		Glioma (n = 35)*			*Meningioma (n = 31)*			*ACN (n = 6)*		
Virus	*Age (years)*	n	*SP (%)*	*(95% CI)*	n	*SP(%)*	*(95% CI)*	n	*SP(%)*	*(95% CI)*
HCMV	20–39	7	42.9	(10, 82)	4	75.0	(19, 99)	3	33.3	(1, 91)
	40–59	13	61.5	(32, 86)	17	58.8	(33, 82)	2	100	(16, 100)
	60–84	15	66.7	(38, 88)	10	80.0	(44, 97)	1	100	(3, 100)
All		35	60.0	(42, 67)	31	67.7	(49, 83)	6	66.7	(22, 96)
HSV	20–39	7	85.7	(42, 100)	4	75.0	(19, 99)	3	66.7	(9, 99)
	40–59	13	100	(75, 100)	17	76.5	(50, 93)	2	100	(16, 100)
	60–84	15	86.7	(60, 98)	10	90.0	(56, 100)	1	100	(3, 100)
All		35	91.4	(77, 98)	31	80.7	(63, 93)	6	83.3	(36, 100)
EBV	20–39	7	100	(59, 100)	4	100	(40, 100)	3	100	(29, 100)
	40–59	13	84.6	(55, 98)	17	94.1	(71, 100)	2	100	(16, 100)
	60–84	15	86.7	(52, 96)	10	80.0	(44, 97)	1	100	(3, 100)
All		35	90.3	(74, 98)	31	85.7	(70, 95)	6	100	(54, 100)
VZV	20–39	7	85.7	(42, 100)	4	50.0	(7, 93)	3	100	(29, 100)
	40–59	13	100	(75, 100)	17	100	(80, 100)	2	100	(16, 100)
	60–84	15	86.7	(60, 98)	10	90.0	(56, 100)	1	100	(3, 100)
All		35	90.3	(74, 98)	31	91.4	(77, 98)	6	100	(54, 100)

*No serum sample available for five glioma patients.
SP, seroprevalence; ACN, acoustic neurinoma; HCMV, human cytomegalovirus; HSV, herpes simplex virus; EBV, Epstein-Barr virus; VZV, varicella-zoster virus.

prevalences of the general population (German population, if possible).

Discussion

For several years, HCMV has been controversially discussed to be involved in the pathogenesis of miscellaneous malignancies (Harkins *et al*, 2002; Cinatl *et al*, 2005; Huang *et al*, 2002; Samanta *et al*, 2003), including human brain tumors (Wrensch *et al*, 1997, 2001). In 2002, it has been reported that all of 27 gliomas investigated expressed multiple HCMV-specific molecules in contrast to tissues from meningiomas, Alzheimer's disease, stroke, and normal brain (Cobbs *et al*, 2002). Therefore, the authors suggested that HCMV might play an active role in brain tumor development.

The present study was conducted to evaluate these results in a larger study population. In none of the 77 brain tumor samples as well as in none of the short-term cultures derived from 4 of these samples, HCMV DNA could be detected by nested PCR analyses. To ensure the validity of the results, we used different PCR protocols for amplifying sequences of a specific HCMV genomic region. In addition, PCR analyses were done on different genomic regions of the viral genome. Additional PCR analyses performed with glioma short-term cultures derived from patients distinct from the study participants also could not trace HCMV DNA. Therefore, a selection bias is unlikely to have appeared in the present study. Moreover, if persisting HCMV DNA would be a prerequisite of maintaining the transformed state of glioma cells, viral DNA sequences should be present in each of the cultured cells, and thus easily being detected by PCR.

It cannot be excluded that previously reported positive results in brain tumor tissues were due to a contamination of the tissue with HCMV-positive blood cells yielding false-positive results in brain tumor tissue. Therefore, in the present study, PCR detecting HCMV DNA in blood samples of the brain tumor patients was performed as a specificity control, but no HCMV DNA was found using different PCR protocols.

In the analyses of Cobbs *et al* (2002), HCMV PCR was negative in two glioma samples although immunohistochemistry clearly showed the presence of several HCMV proteins. These discrepancies are known to be common for HCMV detection in different tissues (Gass *et al*, 1993; Knosel *et al*, 2004). Therefore, immunohistochemistry was performed in the present study to detect HCMV-specific proteins. As for HCMV DNA detection, different protocols were used to control for false-negative results. Furthermore, immunohistochemistry was performed using both tissue sections and short-term cultures derived from four of the participants' tumor tissues. The presence of immediate early-1 (IE1) or early protein would have been indicative for reactivation or latency of HCMV, whereas the pp65 protein is the target antigen for rapid diagnosis of HCMV clinical infections (Landolfo *et al*, 2003; RKI, 2000a). However, none of the three proteins investigated were detected in brain tumor tissues or in brain tumor short-term cultures. Unfortunately, there was no HCMV protein-positive glioma tissue available as a control, and we used HCMV antigen-positive lung tissue. This might not be the ideal control but in the report of Cobbs *et al* (2002), a positive control is not described. In addition, the lack of detection of proteins is somewhat expected in view of the lack of corresponding DNA sequences, as demonstrated above.

It is not clear if the conflicting results between the study of Cobbs *et al.* (2002), which included tissues from US citizens, and the present study may be due to geographical differences in the prevalence of HCMV. However, HCMV molecules were also not detected in primary brain tumors and in several other malignancies in a very recent US study (Lau *et al*, 2005). In this study, besides other cancers, 22 gliomas were investigated for the presence of HCMV by PCR and immunohistochemistry partly using the same protocols as Cobbs *et al* (2002). Similarly, Sabatier *et al* (2005) could not confirm an association between HCMV and primary brain tumors. Therefore, considering the results of these two studies (Sabatier *et al*, 2005; Lau *et al*, 2005) and the results obtained in the present study, the hypothesis of an association between HCMV and the development or progression of primary brain tumors can not be supported.

HCMV, however, is not the only member of the family of herpesviruses controversially discussed to be involved in brain tumor pathogenesis. Therefore, the seroprevalences of IgM and IgG antibodies to HCMV, HSV, EBV, and VZV were evaluated.

No IgM antibodies to any of these herpesviruses were found, indicating an absence of an acute herpesvirus infection in the brain tumor patients. The overall seroprevalence of IgG antibodies to HCMV in the study participants was similar to a report of the Robert-Koch Institute, Germany (RKI, 2000a). A study evaluating the HCMV seroprevalence in a study population aged 20 to 40 years in the Freiburg area (Southern Germany) reported a seroprevalence for HCMV of 42% (Krech, 1973). Age-stratified analyses in the subjects of the present study yielded a similar seroprevalence.

In the present study, the overall seroprevalence found for IgG antibodies to HSV in brain tumor patients was 86% (95% CI 76%–93%). This is in the line with recent data on seroprevalences in the German population reported by Hellenbrand *et al* (2005) and Wutzler *et al* (2000). In addition, stratification by age (below and above 40 years of age) in the present study resulted in prevalences similar to Rabenau *et al* (2002). In their survey, the seroprevalences of IgG antibodies to HSV in the Frankfurt am Main area, Germany, were 64% for men and 71% for women in the age group 15–39 years. Above 40 years, prevalences were 83% and 85%, respectively. In analyses

stratified by tumor type in the present study, an increase in the HSV seroprevalence in glioma patients compared to aforementioned publications could be observed.

The evaluation of IgG antibodies to VZV in brain tumor patients showed a similar seroprevalence as those found in the German population by Wutzler *et al* (2001) and the Robert-Koch Institute, Germany (RKI, 2000b). Stratification by tumor type, too, yielded seroprevalences similar to the overall value.

EBV is able to infect astrocytes *in vitro* (Menet *et al*, 1999). Therefore, the seroprevalence of antibodies to EBV in the study participants was assessed. According to Cohen (2000), IgG antibodies to EBV have an overall seroprevalence of >90% in the adult population, which is in the line with obtained prevalences in the present study, indicating that no differences were present in the EBV prevalence of brain tumor patients compared to the general population.

The seroprevalence of IgG antibodies to HCMV, HSV, EBV, and VZV in glioma patients compared to controls had been evaluated by Wrensch *et al* (1997, 2001). An inverse correlation of anti-VZV and anti-EBV antibodies and glioblastoma was reported. A similar trend could be observed for anti-EBV antibodies and glioblastoma in the present study (data not shown); however, overall EBV and VZV seroprevalences of the study participants were similar to those reported for the general population. No positive association for the occurrence of anti-HCMV antibodies and primary brain tumors was observed. Tumor type–specific analyses in the study of Wrensch *et al* (2001) yielded a higher seroprevalence of IgG antibodies to HSV in WHO grade IV gliomas (glioblastoma multiforme), suggesting that HSV may be involved in glioma pathogenesis. A slight increase in HSV IgG seroprevalence could also be observed in the present study, either for all glioma subtypes combined or for glioblastomas alone (data not shown), being in the line with the above-mentioned study.

Summarizing the results obtained from the serological analyses, no significant differences between seroprevalences in brain tumor patients and the general population could be observed. There seems to be a tendency towards a positive association of anti-HSV antibodies and glioma, but the sample size in the present study is too small to clearly establish this.

The results of Cobbs *et al* (2002) reporting that HCMV genes and proteins were present in all of glioma tissues investigated could not be confirmed. In contrast, the present study is in the line with the more recent studies of Lau *et al* (2005) and Sabatier *et al.* (2005), who also could not find HCMV molecules in brain tumor tissues. Therefore, the hypothesis of an association between HCMV and the development or progression of primary brain tumors cannot be supported.

Material and methods

Sample collection

Between December 2002 and March 2004, 77 brain tumor samples were obtained from the Department of Neurosurgery, University of Heidelberg, and immediately stored at −80°C until further preparation. In total, tissues from 40 gliomas, 31 meningiomas, and 6 ACNs were collected. Additionally, a corresponding heparinized blood sample was collected from 94% of these brain tumor patients (35 gliomas, 31 meningiomas, 6 ACN).

Nested PCR of tumor tissues

DNA from all brain tumor samples was purified from fresh-frozen surgical specimens using High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

HCMV DNA sequences were amplified by nested PCR using primers specific for the HCMV glycoprotein B (gB) (UL55) gene, which encodes one of the most highly conserved herpesvirus-common proteins (Chee *et al*, 1989) (primer EL: 5′-TAA CGG GTA CTG TGG GTG TTG G-3′; primer ER: 5′-ACC AAG TAC CCC TAT CGC GTG T-3′; primer IL: 5′-CTG CCC AGC AGA TAA GTG GTG T3-′; primer IR: 5′-ATC ATC TGC ACC TCG ATG AAG C3-′). 0.2 μl of the primers EL and ER and 2 μl of template DNA were added to 45 μl of PCR Supermix (Life Technologies, Eggenstein, Germany) and PCR was carried out for 35 cycles. Subsequently, 1 μl of the reaction mix was added to a second mix containing 0.2 μl of the primers IL and IR and 45 μl PCR Supermix, and PCR was continued for another 35 cycles. Primer annealing occurred at 67°C for 45 s, DNA extension at 72°C for 45 s, and DNA denaturation at 94°C for 45 s. Primers EL and ER amplified a 419-bp product, primers IL and IR a 167-bp amplimer.

As it is known that the occurrence of false-negative results is quite common in PCR tracing HCMV (Gass *et al*, 1993), an additional PCR protocol amplifying sequences of another HCMV gene, namely the HCMV-specific IE1 gene, was performed. In this nested PCR, the following primers were used: primer EXTL: 5′-CGA GGC TAC GCT TCC TAC AC-3′; primer EXTR: 5′-GCG TAC GAG GAA CTC TTT GC-3′; primer INTL: 5′-GAC GAC CCT TTC GAT GAG TG-3′; primer INTR: 5′-GCC CAA CAA CTG GTG GTA AC-3′. 0.2 μl of primers EXTL and EXTR and 2 μl of template DNA were added to 45 μl of PCR Supermix and PCR was carried out for 30 cycles. Afterwards, 1 μl of the reaction mix was added to a second mix containing 0.2 μl of primers INTL and INTR and 45 μl PCR Supermix, and PCR was continued for another 30 cycles. Primer annealing occurred at 64°C for 45 s, DNA extension at 72°C for 45 s, and DNA denaturation at 94°C for 30 s. Primers EXTL and EXTR amplified a 246-bp product, primers INTL and INTR a 177-bp amplimer.

In addition, a subset of brain tumor DNA samples was tested by nested PCR using specific primers for the HCMV IE1-72 and gB (UL55) genes as described by Mangano *et al.* (1992) and Cobbs *et al* (2002), respectively.

For each PCR, a positive control (DNA from a cell lysate of HCMV-infected cells) and a negative control (double-distilled water) were run. Sensitivity of the PCR analyses was ensured by amplifying samples of the positive control DNA that had been serially diluted in glioma DNA extracts. Amplified DNA products were separated through agarose gel with ethidium bromide and visualized under UV light.

Sequences of the cellular GAPDH gene were successfully amplified for all samples (primers GAPDH1: 5′-TTA ACT CTG GTA AAG TGG ATA TTG TTG CCA-3′, and GAPDH2: 5′-TAT TTG GCA GGT TTT TCT AGA CGG CA-3′), demonstrating that no inhibitors of the PCR were present in the DNA samples.

Nested PCR of patients' blood samples
To exclude a possible contamination of the brain tumor tissues with HCMV DNA–positive blood cells, different nested PCRs were performed with DNA from corresponding blood samples. DNA was extracted from patients' blood samples using the REDExtract-N-Amp Blood PCR Kit (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) according to the manufacturer's instructions. Afterwards, DNA was amplified by nested PCR using primers specific for the HCMV IE1 gene as described by Mangano *et al* (1992) and, in a second protocol, primers specific for gB (UL55) as described by Cobbs *et al* (2002).

Nested PCR of brain tumor short-term cultures
Additional PCR analyses were carried out with low-passage short-term cultures (prepared as previously described by Herrero *et al*, 2004) of one glioma (unknown grading), two meningiomas (WHO grade I), and one ACN derived from study participants' tumor tissues. In addition, low-passage short-term cultures of four glioma samples collected prior to the present study period (three gliomas WHO grade III, one glioblastoma WHO grade IV) were tested. After DNA extraction using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, all cultures were analyzed by PCR using primers specific for HCMV-specific IE1 gene as used by Cobbs *et al* (2002).

Immunohistochemistry of brain tumor samples
To analyze the presence of HCMV-specific proteins in brain tumor samples, surgical specimens of 38 gliomas, 29 meningiomas, and 6 ACNs obtained in paraffin blocks were cut (8 μm), mounted on SuperFrost Plus slides (Menzel Gläser, Braunschweig, Germany), dewaxed with xylene, and gradually hydrated. Sections were blocked for endogenous peroxidase (3% H_2O_2, 10 min), avidin, and biotin (BioGenex, San Ramon, USA; each for 15 min). Antigen retrieval was achieved by cooking in 0.01 M citrate buffer pH 6.0 (BioGenex). Immunohistochemistry was performed using three different HCMV-specific monoclonal antibodies: anti-pp65 (clones 2 and 6; Novocastra, Newcastle upon Tyne, United Kingdom, dilution 1:200), which is specific for the HCMV pp65 protein, anti-EA (clone QB1/42; Novocastra; dilution 1:100), which detects an HCMV early antigen (EA) and anti-IE (Chemicon, Temecula, USA; dilution 1:100), which reacts with an IE nonstructural HCMV protein of 68 to 72 kDa. Detection took place using a conventional horseradish peroxidase system (BioGenex). AEC (Dako, Carpinteria, USA) served as chromogen. Finally, the slides were briefly counterstained with hematoxylin and mounted aqueously.

Immunohistochemical analyses were performed blinded for tumor type. Because no HCMV-positive brain tissue could be obtained, HCMV-positive human lung sections (Dako) were used as a positive control.

Immunohistochemistry of brain tumor short-term cultures
To exclude that the presence of paraffin or other factors in brain tumor tissues interfered with the detection of HCMV in immunohistochemistry, short-term cultures were prepared from some of the primary brain tumors (two meningiomas WHO grade I, one glioma with unknown grading, one astrocytoma WHO grade III). Cultured glioma cells as well as meningioma cells were grown on cover glasses and fixed with methanol and acetone (5 min at -20°C each). Because HCMV seems to be stable only during limited passage in cell culture (Mocarski, 2002), immunohistochemistry was carried out with brain tumor cultures not exceeding passage 10. The monoclonal anti-pp65 antibody (clones 2 and 6, dilution 1:200; Novocastra) and a conventional horseradish peroxidase method (BioGenex) were used. Human skin fibroblasts infected by HCMV strain AD169 served as positive controls.

Serology
Serum samples were obtained from 72 (94%) of the 77 study participants. Five glioma patients did not provide a blood sample. Sera were investigated for the presence of anti-HCMV, anti-VZV, anti-EBV, and anti-HSV IgM and IgG antibodies using an enzyme-linked immunosorbent assay (Enzygnost; Dade-Behring, Marburg, Germany) at the Department of Virology at the University of Heidelberg, utilizing the automatic BEP-III-System (Dade-Behring).

Statistical analyses
Descriptive statistics were computed using the PROC FREQ procedure of the statistical software package SAS. Seroprevalences of IgG antibodies to HCMV, HSV, EBV, and VZV were calculated. Corresponding exact 95% confidence intervals (CIs), which cover the true value in the respective population with a probability of 95%, were assessed.

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