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Propagation of archetype and nonarchetype JC virus variants in human fetal brain cultures: Demonstration of interference activity by archetype JC virus

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In immunologically normal individuals, the polyomavirus, JC virus (JCV), produces an asymptomatic primary infection followed by lifelong persistence of the virus in renal tubular epithelial cells. In some immunocompromised patients, however, in particular acquired immunodeficiency syndrome (AIDS) patients, JCV causes an opportunistic central nervous system (CNS) disorder, progressive multifocal leukoencephalopathy (PML). JCV DNA as it persists in kidneys (archetypal JCV) and JCV DNA isolated from PML lesions show differences in their regulatory regions in which transcription and replication are controlled. Archetypal JCV DNA has a single enhancer and no rearrangements or deletions in the regulatory region. In contrast, JCV DNA from PML isolates is characterized by alterations in the regulatory region. Some PML-associated JCVs can be grown in cultures of human fetal brain (HFB) cells. Growth of archetypal JCV in cultured cells has not been reported, however. Here we demonstrate successful propagation of the archetypal JCV, strain GS/K, in HFB cells. Growth occurred more slowly and to lower titers than is seen with the prototypical PML JCV strain Mad-1, with relatively few cells containing viral T antigen (T-Ag) or viral capsid protein, Vp1. Interestingly, GS/K growth could be enhanced, with a large increase in viral DNA and cytopathic effect, by coinfection with GS/B, a nonarchetypal brain-derived JCV variant isolated from the same PML patient as GS/K. The amount of GS/K DNA was also greatly enhanced when it was cotransfected with Mad-1 JCV DNA, the prototypical PML isolate. In contrast to GS/K plus GS/B–cotransfected cells, in GS/K plus Mad-1–infected cells, cytopathic effect was not increased. On subsequent passage of culture lysates to naïve cells, however, the infection produced by either combination of viral DNAs slowed, no cytopathic effect (CPE) was present, and the amount of GS/B or Mad-1 viral DNA was greatly reduced as compared to that of GS/K DNA. These data suggest that GS/K was able to use either GS/B or Mad-1 as a helper and that GS/K was in turn able to interfere with the growth of either helper virus. Archetype JCV can be successfully propagated in HFB cells, although infection develops much more slowly than that caused by the PML JCV variant Mad-1. The ability of archetypal and variant JCVs to enhance or retard each other's replication may have implications *in vivo* for the maintenance of JCV persistence and the growth of JCV variants. *Journal of NeuroVirology* (2003) **9,** 567–576.

Keywords: archetype JCV; growth of JCV; human spongioblasts; JC virus; PML; polyomavirus; polymerase chain reaction; progressive multifocal leukoencephalopathy; viral regulatory region

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Introduction

JC virus (JCV) is a human polyomavirus that is a ubiquitous agent in humans (Padgett and Walker, 1973; Brown *et al*, 1975; Bofill-Mas *et al*, 2000;). In immunologically normal persons, JCV causes an asymptomatic primary infection followed by lifelong persistence of the virus in kidneys and possibly other organs (Agostini *et al*, 1998; Markowitz *et al*, 1993). In immunocompromised patients, however, JCV may produce a fatal, opportunistic central nervous system (CNS) infection, progressive multifocal leukoencephalopathy (PML), characterized by lytic infection of oligodendrocytes with secondary demyelination, and by nonproductive infection of astrocytes (Zu Rhein, 1969). PML is particularly associated with acquired immunodeficiency syndrome (AIDS), such that 5% of patients with AIDS may die from PML (Agostini *et al*, 1997a).

JCV isolated from persistently infected kidneys and from urine is believed to be the form of the virus transmitted in nature and is termed "archetypal" JCV (Agostini *et al*, 1998; Boldorini *et al*, 2001; Yogo *et al*, 1990). Archetypal JCV DNA has a single enhancer and no rearrangements or deletions in the regulatory region. In contrast, strains of JCV recovered from PML brains almost invariably have alterations in the viral regulatory region. These include duplications of the enhancer and sometimes other regulatory region elements (Agostini *et al*, 1998; Iida *et al*, 1993). The nonarchetype may also contain deletions of varying sizes in regulatory region elements outside the enhancers (Agostini *et al*, 1998; Boldorini *et al*, 2001; Tominaga *et al*, 1992). The DNA sequence of the archetypal regulatory and coding regions vary only slightly in some strains isolated from different parts of the world, and are often identical (Cubitt *et al*, 2001). However, JCV regulatory region sequences of archetype versus nonarchetype JCV may vary considerably (Ault and Stoner, 1993).

The close association of nonarchetypal JCV strains with PML has led to the hypothesis that during persistent infection by archetype JCV in kidneys or other tissues, variants may arise in which the enhancer element is duplicated and/or other alterations appear in the regulatory region, and that these variants may enjoy selective advantage in terms of growth or tissue tropism. In immunologically normal individuals, these variants are readily contained by the host immune response. In AIDS or other states of impaired immunity, however, these variants may spread to the brain and cause PML. Little data exist, however, as to how these variant strains evolve from the archetype, which variant strains might enjoy a growth advantage, and whether the interaction of archetype and nonarchetype strains affects viral growth. These questions have proven difficult to address, however, because the JCV archetype has been considered difficult to grow in cultured cells not containing T antigen (T-Ag), even in human fetal brain (HFB) cultures, which are highly permissive for Mad-1 (Ault, 1997; Daniel *et al*, 1996; Sock *et al*, 1996). Archetype JCV has been propagated in Cos 7 cells, but these cells contain simian virus 40 (SV40) T-Ag, which may stimulate replication of the archetype genome (Hara *et al*, 1998). In the present study we demonstrate successful propagation of the archetypal JCV strain, GS/K in HFB cells maintained for long periods of time. We also demonstrate that coinfection of HFB cells with GS/K and either of two PML variant JCV strains significantly alters viral growth.

Results

Virus growth

Transfection of HFB cells with Mad-1 resulted in the presence of T-Ag in 12% of cells by day 10 and 81% of cells by day 21 (Table 1). Cytopathic effect (CPE) appeared by day 17 and was extensive by days 24 to 30. In contrast, transfection of GS/K or GS/B DNAs into HFB 271 and HFB 558 cells did not result in detectable CPE, even in cultures maintained for 4 months. Immunofluorescence staining of GS/K- and GS/B-infected cultures at 3 to 4 weeks following transfection demonstrated positive staining for T-Ag and Vp1 protein in 0.1% to 1% of cells (Figure 1, Table 1). However, the percentage of cells showing viral proteins gradually increased, so that 31% of cells in GS/K-infected cultures were positive after 3 months (Table 1). After 3 months, 21% of the cells in GS/B-infected cultures contained T-Ag and 11% of the cells had Vp1 (Table 1). GS/K-infected cultures were maintained for over 11 months. By 8 to 10 months after inoculation, 34% of the cells were T-Ag positive.

Analysis of virus growth

Several methods were used to determine the relative growth advantage of archetype JCV GS/K in the presence and absence of GS/B or Mad-1. In the first method, we assayed the amount of viral DNA in infected cells after Hirt extractions (Table 2). This demonstrated that, relative to SV40- and Mad-1 JCV–infected HFB cells, GS/K or GS/B produced little viral DNA. However, in GS/K + GS/B– or GS/K + Mad-1–coinfected cells, much greater amounts of viral DNA were produced initially. This increase did not persist, as after serial passage, viral production fell to somewhat lower levels (Table 2).

In the second method, serial dilutions of lysates from infected cells were assayed for infectivity, using T-Ag detection as the final parameter, as described in the Materials and methods. The results from these experiments indicated the following: (a) In cultures infected with Mad-1 or coinfected with GS/K + GS/B and maintained for 25 to 30 days, 1×10^7 infectious units of virus per ml of lysate were present (Table 3). (b) In contrast, cultures infected with only GS/K and assayed after 4 to 12 weeks, usually contained

Table 1 Immunofluorescence analysis of T antigen and Vp1 in JCV-infected HFB cells

Cells	*Virus*	*Days postinfection*	*% T antigen–positive cells*	*% T-Ag fluorescence intensity*	*% Vp1-positive cells*
HFB 271	GS/K	10	1	+	0.5
HFB 271	GS/K	21	4	++, +	2
HFB 271	GS/K	30	8	++, +	4
HFB 271	GS/K	93	31	++, +	14
HFB 271	GS/K	215	34	++, ++, +	18
HFB 558	GS/K	10	0.1	+	0.1
HFB 558	GS/K	35	0.1	+	0.1
HFB 558	GS/K	61	5	+	2
HFB 558	GS/K	93	7	+, ++	3
HFB 558	GS/K	159	20	+, ++	11
HFB 271	GS/B	10	4	+	2
HFB 271	GS/B	29	10	+	4
HFB 271	GS/B	61	14	+, ++	10
HFB 271	GS/B	91	21	+, ++	11
HFB 271	GS/K + GS/B	16	61	++	22
HFB 271	GS/K + GS/B	29	86	+++	43
HFB 271	Mad-1	10	12	+, +++	11
HFB 271	Mad-1	21	81	+++, ++++	61
HFB 271	Mad-1	28	93	++++	44
HFB 271	GS/K + Mad-1	10	8	+	3
HFB 271	GS/K + Mad-1	16	16	++, +++	8
HFB 271	GS/K + Mad-1	21	86	++, +++	29
HFB 271	GS/K + Mad-1	32	91	++, +++, ++++	39

Fluorescence intensity: + = very weak; ++ = weak to moderate; +++ = robust; ++++ = very robust.
In the Vp1 assay, the great majority of cells that were positive showed robust staining.

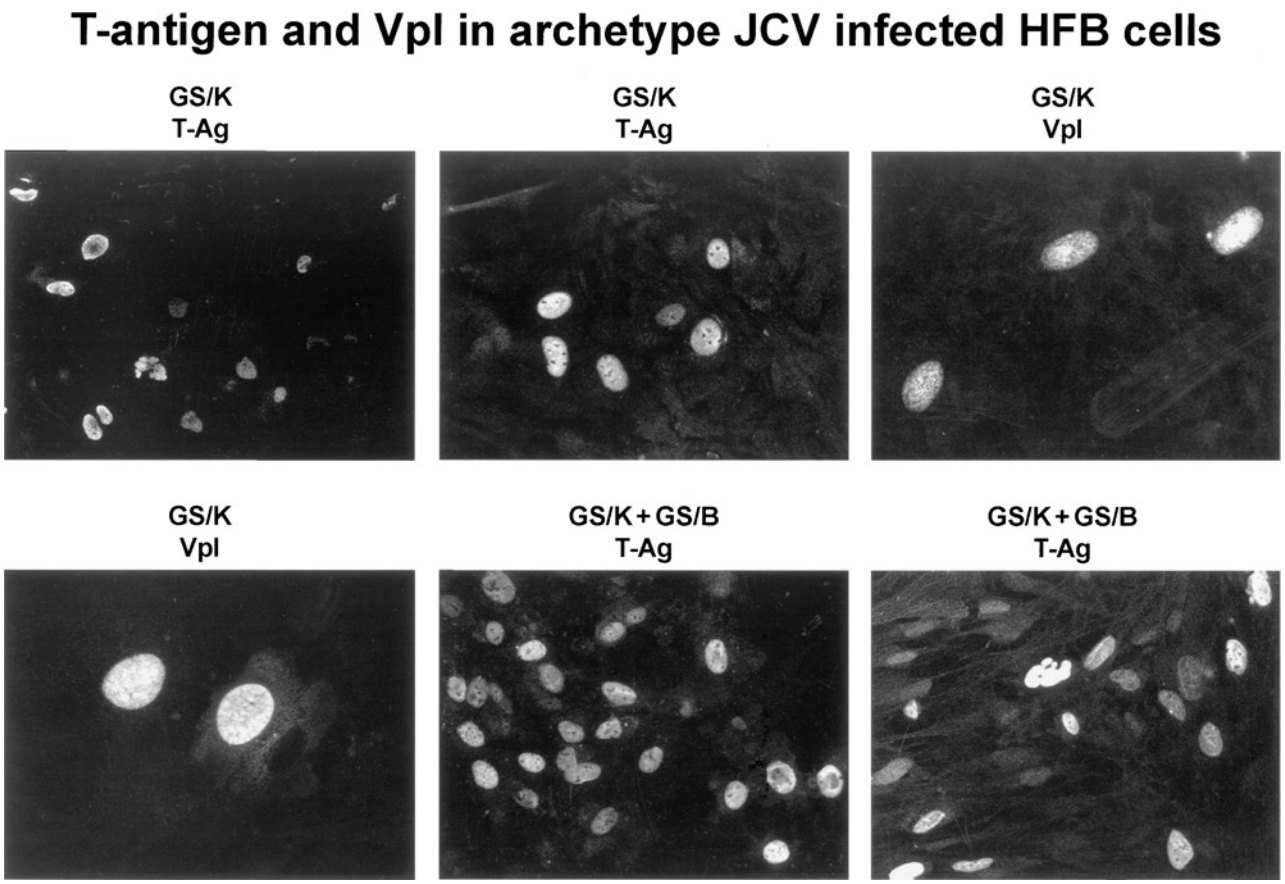

Figure 1 T antigen (T-Ag) and Vp1 antigen expression in archetype JCV-infected cells. In these experiments, HFB cells infected with GS/K alone show expression of both T-Ag and Vp1 in only a minority of cells. In contrast, much larger numbers of cells are shown to express T-Ag in cultures coinfected with GS/K + GS/B. The figure shows duplicate experiments of GS/K alone and GS/K plus GS/B.

Table 2 Quantitation of viral DNA in infected HFB cells

Virus	*JCV DNA (ng/ml)*	*Passage no.*
SV40	50–70	1–2
JCV: Mad-1	20–25	1–3
JCV: GS/K	2–12	1–3
JCV: GS/B	1–9	1–3
GS/K + GS/B	18	After cotransfection
GS/K + GS/B	8–14	After 1 passage
GS/K + GS/B	1–5	After 2 passages
GS/K + Mad-1	25	After cotransfection
GS/K + Mad-1	5	After 1 passage

a maximum of 10,000 infectious units per ml. In GS/B infections, there appeared to be a maximum of only approximately 1000 infections per cell (Table 3). Consequently, both GS/K and GS/B grew relatively poorly as compared to GS/K + GS/B–coinfected cells, and to Mad-1.

In the third method, lysates from infected cells were subjected to serial dilution and the DNA in each dilution was purified and subjected to the polymerase chain reaction (PCR). The results from these analyses (not shown) indicated that SV40 propagated to the highest titer, Mad-1 and GS/K + GS/B were next, then GS/K, and GS/B had the lowest titer. These finding were consistent with assays 1 and 2 and also with the frequency of T-Ag–positive cells in the various infections (Table 1). The higher the frequency of T-Ag–positive cells, the greater the amount of viral DNA and virus was produced.

PCR analysis of viral DNA in GS/K-infected HFB cells

JCV DNA in cells infected with GS/K or GS/B was detected by PCR analysis using primers for the viral regulatory region. This was done from extracts prepared from cells infected with GS/K or GS/B at or near the end of each passage, which was every 2 to 3 months. We also determined the relative amounts of GS/K and GS/B or GS/K and Mad-1 in coinfected cells. We confirmed by DNA sequencing that the viral regulatory region present in GS/K-infected cells was, as expected, archetypal (data not shown). Similarly, GS/B-infected cells contained the nonarchetypal GS/B regulatory region, with a duplication of both the regulatory region elements and the agno gene from the 5′ terminus of the JC viral late region. Molecular cloning and sequencing of the GS/K regulatory region in viral DNA extracts after only one passage demonstrated no mutations in the GS/K regulatory region, indicating that GS/K growth in HFB cells did not require regulatory region modification. We found, however, that variants appeared after one additional passage or in GS/K long-term persistently infected HFB cells (Figure 2). The variants usually migrated ahead of that of the GS/K molecular clone (Figure 2, lanes 2, 4, 5, and 7 to 10). These variants have not yet been cloned and sequenced.

Table 3 JC virus infectivity in human fetal brain cultures

Virus	*Titer*
SV40	5×10^7
JCV: Mad-1	1×10^7
JCV: GS/K (archetype)	1×10^4–1×10^5
JCV: GS/B (brain-derived variant)	1×10^3–1×10^4
JCV: GS/K + GS/B	1×10^7

Coinfection with GS/K plus GS/B and with GS/K plus Mad-1

Originally, we had expected to find that infection with GS/K was abortive and that no T-Ag or Vp1 would be produced. However, we hypothesized that some nonarchetypal JCV variants would propagate and, if so, that they might stimulate growth of the archetype GS/K. Consequently, we performed experiments in which GS/K and GS/B were cotransfected into HFB cells or where cells were first transfected with GS/K or GS/B and then later superinfected with potentially complementing GS/B or GS/K. Cultures coinfected with GS/K plus GS/B exhibited extensive CPE by 4 to 5 weeks after infection, and the amount of viral DNA in the Hirt supernatant was greatly increased such that there was a visible band in the CsCl gradient after ultracentrifugation. Also, immunfluorescense analysis of T-Ag and Vp1 demonstrated a very large increase in the frequency of T-Ag– and Vp1-positive cells. In GS/K- or GS/B-infected HFB cells, usually only 1 to 3 ng of viral DNA per ml was detected. However, in GS/K + GS/B–coinfected cells, 18 ng/ml of viral DNA was detected (Table 2). These finding are consistent with the larger amount of infectious virus produced in coinfected cells. Similarly, in GS/K + Mad-1–coinfected cells, as great as 25 ng of viral DNA per ml was found (Table 2). When T-Ag and Vp1 were analyzed 4 to 5 weeks after transfection with either GS/K or GS/B alone, less than 10% of the cells contained T-Ag and less than 5% contained Vp1 (Table 1). In contrast, in cotransfected cells, as many as 86% of the cells had T-Ag and 43% contained Vp1. These levels were not observed in singly infected cells maintained in culture for several months. In addition, control cultures infected with either virus alone did not exhibit CPE. This acceleration of virus growth and CPE did not occur when cells were first infected with one of the viral DNAs and subsequently superinfected by the second potentially complementing viral DNA (Figure 3). For the first one or two serial passages of virus recovered from simultaneously coinfected HFB cells, we found that smaller amounts of viral DNA were produced and that CPE did not result (Table 2). However, after additional virus passages, CPE reappeared and greater amounts of virus were produced (not shown). This suggested the development of recombinant viral genomes possessing greater cytopathogenicity. These putative new variants are being cloned.

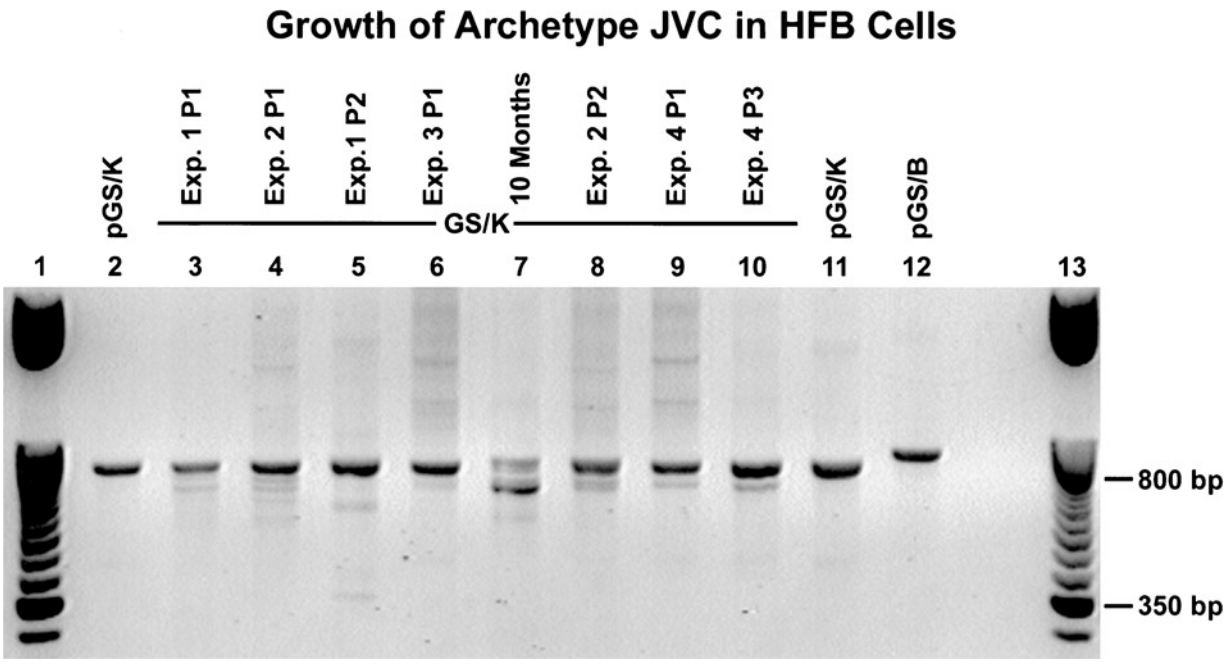

Figure 2 PCR analysis of viral DNA from GS/K-infected HFB cells. In four independent experiments, GS/K DNA was transfected into HFB271 or HFB 558 cells and virus was recovered from the cells 60 to 75 days later. It was passed into fresh naive cells and then passed again 60 to 75 days later. Viral DNA was recovered from infected cells maintained in replicate 25-cm^2 tissue culture flasks, and analyzed by PCR as described in Materials and methods. Lanes 1 and 13: 50-bp size standards; lane 2: plasmid pGS/K DNA; lanes 3 to 5: GS/K regulatory region DNA recovered 2 to 3 months after independent transfection or infection of HFB271 cells; lane 5: regulatory region DNA recovered 2 months after the beginning of the second virus passage; lane 6: passage 1, experiment 3 of GS/K-infected HFB271 cells (compare to lane 4); lane 7: GS/K in HFB558 cells, 10 months, virus-producing cells maintained in culture but neither virus nor cells were passed before 10 months; lane 8: HFB558, same experiment as lane 7 but DNA extracted after only 7.5 months; lanes 9 and 10: HFB271 cells, passage 2 of independent GS/K infections in both lanes; lane 11: GS/K in HFB271 cells, virus passage 1 (total infection period of 7.5 months); lane 12: GS/B plasmid. Note the viral DNA variants moving ahead of GS/K in small amounts in lanes 3 to 5 and 8 to 10 but in relatively large amounts in lane 7.

We then cotransfected cells with GS/K and Mad-1 DNAs. In these cultures, CPE occurred within 3 to 4 weeks, and nearly all the cells died. However, when the virus from these cells was passed to naïve cells, the appearance of CPE was delayed for more than 5 weeks and was less extensive, with the apparent destruction of fewer cells. At a subsequent passage, CPE was delayed until 6 to 7 weeks and was then mild.

Analysis of viral DNAs

PCR analysis of the viral regulatory region from viral genomes recovered from coinfected cells at the end of the period of the first cotransfection (4 to 5 weeks) showed that although the amount of viral DNA was greatly increased, there was a near-equal representation of both viral regulatory regions (Figure 3, lane 3). However, after passage of the virus from coinfected cells, the archetype became the predominant species and the total amount of viral DNA was reduced to lower levels, although somewhat greater than in GS/K- or GS/B-infected cells (Figure 3, Table 2). Within two passages, the GS/B regulatory region could no longer be detected by PCR analysis without the use of GS/B-specific primers (not shown). This apparent dominance by GS/K also occurred, perhaps even more quickly, when GS/B was introduced into cells already infected with GS/K (Figure 3, lane 9). When cells were first transfected with GS/B followed by GS/K a month later and the coinfected cells cultured for four additional weeks, the GS/B regulatory region was the dominant species (Figure 3, lanes 6 and 7). However, after one passage of virus to naïve HFB cells, the GS/K DNA appeared to be more abundant than GS/B, although the latter was still clearly visible (Figure 3, lane 8).

These observations suggested that archetype GS/K and nonarchetype GS/B virus could complement one another to accelerate infectivity, but that GS/K also exhibited interference activity against GS/B, which manifested itself after passage of the virus to fresh cells. An alternative explanation for these findings, however, would be that GS/K simply grew more rapidly and to a higher levels than GS/B, even in coinfected cells. In order to examine this possibility, we coinfected HFB cells with GS/K and Mad-1. Mad-1 produces several thousand fold more virus than GS/K, produced virus more quickly, and is highly cytopathogenic (Table 3). In addition, many more cells express T-Ag (Table 1). If GS/K interferes with GS/B simply because it grows more quickly and to higher titer, then one would expect Mad-1 to dominate GS/K. Cells were transfected with Mad-1 plus pUC19

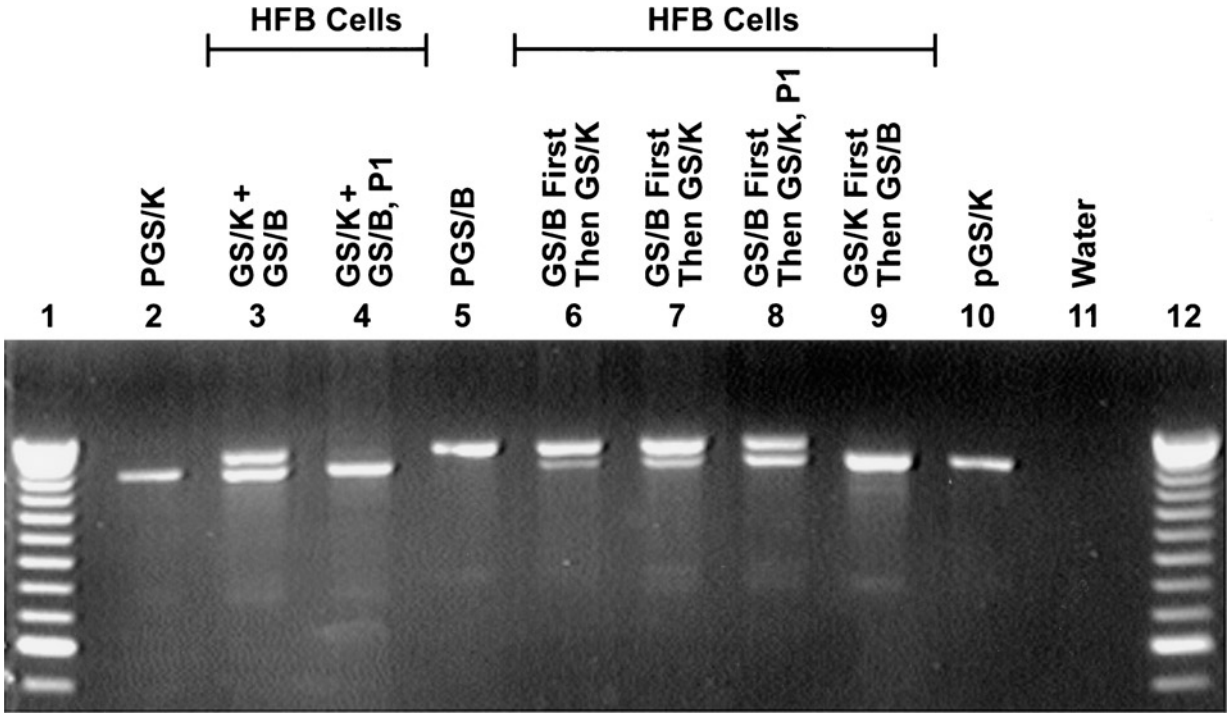

Figure 3 Growth competition between archetype GS/K and nonarchetype GS/B viruses in HFB cells. HFB cells were transfected with GS/K and GS/B using three methods: (1) One microgram each of GS/K and GS/B DNA were cotransfected into HFB cells and the cells were maintained for 4 weeks when extensive CPE appeared. At this time, the viral DNA was recovered and analyzed. In addition, virus was passed to fresh naive cells, whereupon no CPE was observed and then viral DNA was recovered from these cells periodically. (2) In this method, GS/B DNA was transfected into HFB cells and 3 weeks later GS/K was transfected. (3) In the final method, GS/K was transfected first and 3 to 4 weeks hence GS/B was transfected. In both of the latter two procedures, no CPE was observed. Consequently, viral DNA was extracted 6 to 8 weeks later and virus was serially passed as with cotransfected cells. The recovered viral DNAs were analyzed by PCR followed by electrophoresis in agarose gels to identify each of the two viral regulatory regions. The two regulatory regions migrated to different positions on the gel. Lanes 1 and 12: 50-bp DNA size standards; lane 2: pGS/K DNA; lane 3: GS/K + GS/B cotransfected cells, 5 weeks; lane 4: GS/K + GS/B coinfected cells, virus passage 1; lane 5: pGS/B; lane 6: GS/B delivered first, then GS/K delivered 4 weeks later. Viral DNA was extracted and PCR amplified after an additional 4.5 weeks; lane 7: repeat experiment of that shown in lane 6, except that GS/K was delivered 2 weeks after GS/B; lane 8: experiment from lane 6 where the viral DNA extract was made 37 days after one virus passage; lane 9: GS/K first, 3 weeks later GS/B. Viral DNA extracted after an additional 4 weeks; lane 10: pGS/K; lane 11: water control. Viral regulatory regions were recovered and amplified by PCR using JRR 25 and JRR D primers. Viral regulatory region bands contain approximately 750 bp.

or Mad-1 plus GS/K, and the viral DNA extracted and purified when CPE was extensive, at about 4 to 5 weeks. In cells transfected with Mad-1 plus pUC19 or Mad-1 plus GS/K, CPE appeared 21 to 24 days after coinfection. However, severe CPE was delayed 3 to 4 additional days when GS/K was present. The viral DNAs were then recovered from infected cells. Upon PCR amplification of the viral regulatory regions and Sst I digestion, both viral DNAs were observed (Figure 4). However, there was much more GS/K DNA (Figure 4, lanes 5 to 8). After the virus from these cells was passed into naïve cells, the appearance of CPE was delayed until approximately 5 weeks and PCR analysis and Sst I digestion showed that GS/K was still present in much greater amounts than Mad-1 (Figure 4, lanes 6 to 8). After one additional passage, the appearance of CPE was delayed further until after 8 weeks and although unmistakable, it was mild. Replicate cultures from this experiment were harvested at 24 days and at 31 days post infection and the viral DNA analyzed by PCR and Sst I cleavage. In these studies, at least 90%, of the viral DNA showed a GS/K cleavage pattern (Figure 4, lane 8), indicating that GS/K also exhibits interference activity against Mad-1.

Discussion

JCV infection is believed to be transmitted by the archetype; infection is acquired in childhood or adolescence and is not known to cause symptomatic illness. The virus then persists in kidneys, such that periodic excretion of JCV can be detected in healthy as well as immunocompromised adults (Markowitz *et al*, 1993; Kitamura *et al*, 1994; Stoner *et al*, 1996). In contrast, JCV DNAs identified in brains and leukocytes of immunocompetent patients are usually nonarchetypal, and several of the variants identified have been similar to Mad-1. These observations suggest either that the archetype has evolved into nonarchetypal variants that are more readily infectious in extrarenal tissues, or, conceivably, that individuals

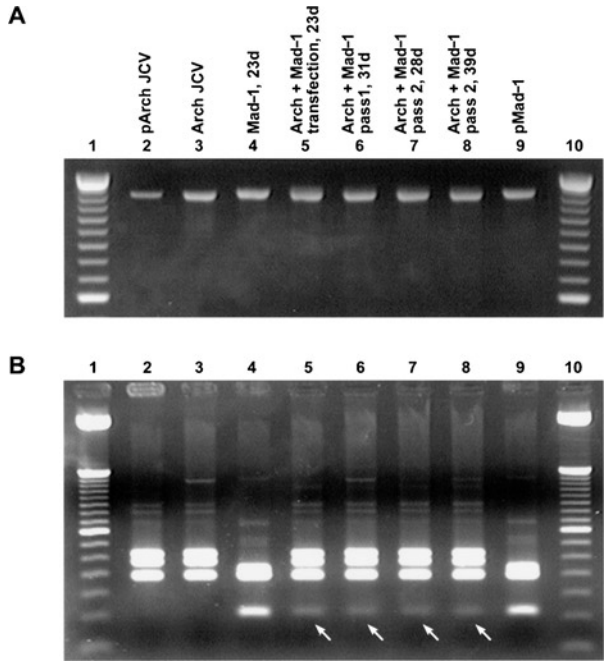

Figure 4 Competitive advantage of GS/K over Mad-1 in HFB271 cells. One microgram each of GS/K and Mad-1 DNAs were transfected into HFB271 cells. In a separate control experiment, Mad-1 was cotransfected with pUC19 DNA. From replicate cultures, the virus was passed at 24 days later (when CPE was extensive) to naive cells and then passed again (second passage) at 31 days when CPE was moderately extensive. At this second passage, CPE failed to appear until 7 weeks later but remained mild. Consequently, the cells could be subdivided twice more. Viral DNA was recovered at 24 days from transfected cells and 29 days after the first passage and then periodically, beginning at 31 days after the second passage. The viral DNA was analyzed by PCR (**A**) and by Sst I or Sph I cleavage of PCR products (**B**). Sph I analyses are not shown but demonstrated again that GS/K inhibited Mad-1 very extensively. (**A**) Lanes 1 and 10: 50-kbp size standards; lane 2, pGS/K regulatory region; lane 3: GS/K from HFB cells 9.5 weeks; lane 4: Mad-1, HFB cells, 23 days; lane 5: GS/K + Mad-1, cotransfected cells, day 23; lane 6: GS/K + Mad-1, passage 1, day 31; lane 7: GS/K + Mad-1, passage 2, 28 days; lane 8: GS/K + Mad-1, passage 2, 34 days; lane 9: pMad-1. (**B**) Sst I digestion of viral DNA in lanes 2 to 9. Lanes 1 and 10, size standards; lane 2: pGS/K; lane 3: GS/K as in **A** (lane 3); lane 4: Mad-1; lane 5: GS/K + Mad-1, same DNA sample as **A** (lane 5); lane 6: GS/K + Mad-1, passage 1, 31 days; lane 7: GS/K + Mad-1, pass 2, 28 days; lane 8: pass 2, 39 days; lane 9: pMad-1.

have become infected a second time but with the nonarchetype (Elsner and Dörries, 1998).

Our studies were initiated because we wanted to develop a system to follow the evolution of the archetype to pathogenic variants. In addition, we wanted to determine if this evolution could occur in cells that did not already contain SV40 T-Ag or JCV T-Ag, either expressed ectopically from plasmids or as in Cos 7 cells, because preexisting T-Ag is presumably not present in cells prior to natural JCV infection. We anticipated that the archetype would be difficult to propagate in tissue culture cells as others have reported (Daniel *et al*, 1996; Sock *et al*, 1996). We had also hypothesized that the nonarchetype might act as a helper for the archetype. One possible mechanism of helper activity would be that in some cells the nonarchetype would produce T-Ag more efficiently, and if the cells were also infected with the archetype, the growth of the archetype would be stimulated by T-Ag. Although this would not explain how one becomes persistently infected with the archetype early in life, it may indicate that a more disseminated archetype infection might occur within a given cell type when the nonarchetypal variants were also present.

In this study, we have demonstrated that the JCV archetype can be grown in HFB spongioblasts. However, viral growth was much slower than that seen with Mad-1, and infection resulted in very little CPE, making detection of virus growth more difficult. Slow growth by the archetype *in vivo*, however, might allow infected cells to remain viable for long periods of time and might allow the archetype to persist by cell-to-cell spread despite the presence of circulating antiviral antibody. Polyomavirus persistence in this manner has been previously documented for the murine polyomavirus, K virus (Greenlee *et al*, 1991). The significantly delayed growth of archetype JCV in HFB cells, with production of minimal CPE, may also explain why archetype JCV growth has been difficult to detect in cultured cells (Daniel *et al*, 1996). The great majority of our studies have been done with the GS/K archetype. However, the type 4 archetype was also found to grow in HFB cells, although perhaps somewhat slower than GS/K (data not shown). This slower growth may be a reflection of experimental variation, the effect of unique nucleotide differences in the GS/K regulatory region, or in the coding region (Yogo *et al*, 1990). We are currently testing several other archetypal JCV strains for growth in HFB cells. An additional explanation for the finding of viral growth is that after GS/K infection of HFB cells, viral mutations may gradually appear and some allow for increased viral growth and perhaps lytic growth. DNA sequence analysis of GS/K DNA recovered after 6 weeks from HFB cells has detected no mutations. However, after longer periods of growth, alterations in some molecules are detected (Figure 2 and unpublished observations). Nevertheless, in either case, GS/K must persist for significant periods because the viral DNA is detectable after many months. In addition, GS/K growth is accelerated by GS/B or Mad-1.

We have shown here that the nonarchetype GS/B can stimulate growth of GS/K and the production of CPE when cells are cotransfected. This stimulation also involves greatly increased production of T-Ag and Vp1. The mechanism of the increase in T-Ag production is not yet understood as neither GS/K or GS/B viral DNAs produce more than 5% T-Ag–positive cells within 4 weeks after transfection. However, the growth stimulation of both viruses is only transitory because after serial virus passage, the GS/K archetype significantly overgrows GS/B, CPE is greatly reduced, and GS/K becomes the predominant species of viral DNA. The ability of GS/K to overgrow GS/B is surprising and dramatic, especially because

the archetype was believed not to grow in tissue culture cells. The ability of the archetype to suppress Mad-1 growth was also surprising, because Mad-1 typically grows far more rapidly and robustly in HFB cells than does GS/K.

Interaction between archetypal and variant polyomaviruses, affecting growth of one or both strains, is not unique to JCV. We have recently demonstrated that SV40 nonarchetypes are generated from infection by archetype SV40 in cultured rhesus kidney cells as well as in human tumor cell lines and in HFB cells (O'Neill *et al*, 2003). As in our experiments with JCV, described above, growth of the nonarchetypes in these cells is significantly inhibited by the archetype. However, when viral lysates from these cells are inoculated into a different cell type, green monkey kidney (GMK) cells, the nonarchetypes were shown to overgrow the archetype SV40. These data indicate that nonarchetypes, at least for SV40, may arise from the archetype in some cell types but that their growth in these cells may be inhibited by the presence of the archetype. In this situation, the nonarchetypes might exhibit a growth advantage only after transfer to another cell type.

The experiments with archetype JCV described here were carried out in the absence of an immune response, unlike the situation which pertains *in vivo*. Nevertheless, our data raise questions as to whether a similar interaction between archetypal and variant JCV strains may occur during natural human infection and may possibly play a role in viral persistence and reactivation. In this scenario, evolution of nonarchetypal strains of JCV from the archetype might initially facilitate growth of the archetype in some cells, leading to urinary excretion of both viruses. This hypothesis would be consistent with the detection of rearranged as well as archetypal JCV DNAs in the urine of some patients (Agostini *et al*, 1998). In addition, potentially pathogenic JCV variants might arise in persistently infected kidneys but be inhibited by the archetype and effectively contained by the host immune response. Immunosuppression might permit spread of both archetypal and nonarchetypal forms into other cell types (e.g., B lymphocytes or oligodendrocytes) in which the nonarchetypes enjoyed greater growth advantage and perhaps greater potential virulence. Further delineation of the pathogenesis of PML will require further study of the interaction of archetype and variant strains of JCV in cultured cells, as well as molecular analysis of JCV strains persisting in brains and extraneural tissues of normal individuals and individuals developing PML.

Materials and methods

Virus

GS/K is an archetypal strain of JCV obtained from the kidney of a patient with PML (Loeber and Dörries, 1988). GS/B is a rearranged variant from the brain of the same patient (Loeber and Dörries, 1988). Recombinant type 4 archetype JCV DNA was a gift of Dr. Gerald Stoner. Mad-1, the initial PML isolate, was a gift from Dr. Peter Howley.

Human fetal brain cells

Brain tissue secured from 16- or 17-week conceptuses under appropriate Institutional Review Board approval was minced with small scissors and the tissue fragments suspended in calcium-, magnesium-, and trypsin-free buffer in a baffled spinner flask. The dispersed cells were collected and suspended in miminal essential medium (MEM) with 5% calf serum containing half the recommended concentrations of kanamycin, streptomycin, ampicillin, and ciprofloxacin (50 μg/ml for K, S, and A, and 5 μg/ml for C) and incubated at 37°C in 25-cm^2 tissue culture flasks. After 4 to 5 days, spongioblast-like cells attached and then formed a monolayer. In some cultures, there was a mixture of spongioblasts and astrocyte-like cells. After several subculturations, the astrocytes usually became difficult to detect. The use of the extra antibiotics was usually essential because in their absence, bacterial contamination often could not be controlled. However, after 7 to 10 days, the cultures were maintained with just kanamycin and streptomycin at the recommended concentrations of 100 μg/ml and no contamination was detected. Three lots of HFB cells were used: HFB 271, HFB 561, and HFB 558; of these, HFB 271 and HFB 558 were used most often. Because these cells did not propagate as continuous cell lines, it was not practical to infect large numbers of cells without ultimately exhausting the overall supply of cells.

Recombinant viral DNA clones

GS/K was cloned in pUC 12, GS/B was cloned in pGEM, and Mad-1 was cloned in pUC 19. Type 4 archetype JCV DNA was obtained as a TOPO XL recombinant. All three were cloned at the EcoR1 sites. The archetype GS/K and the variant GS/B were subjected to DNA sequencing to confirm that their regulatory regions were as reported by Loeber and Dörries (1988). GS/B contained a complex and rearranged regulatory region with a duplicated enhancer, a duplicated 5′ terminus of the Vp1 gene and three small deletions. The Mad-1 strain we used was also sequenced and was found to contain an anomaly, a 10-bp deletion in one of its two enhancers. Otherwise its sequence was as reported by others.

Transfection of HFB cells

We used the calcium phosphate ($CaPO_4$) precipitation method (O'Neill *et al*, 1998) but reduced the Bis buffer concentration to approximately 60% of normal. This helped reduce toxicity often associated

with $CaPO_4$ transfection of human cells. In prior studies with SV40 DNA, we have demonstrated by T-Ag analysis that at least 3% to 5% of the cells were successfully transfected within 3 to 4 days after transfection of viral DNA (unpublished observations).

Serial passage of virus and assays for infectivity
After infection of HFB cells with GS/K or GS/B, part of the supernatant medium along with cells were periodically saved and stored frozen at −70°C. They were then used to initiate a new round of infection. New virus passages were usually made 2 to 3 months after infection with GS/K or GS/B, or 4 weeks after infection with Mad-1.

To test the amount of virus produced in infected cells, media and cells of replicate cultures were frozen and thawed and dilutions of the lysates were used to infect naïve duplicate cultures of HFB cells. After 2 weeks, these newly infected cultures were distributed into dishes with square cover glasses. After another 15 days, these were assayed for T-Ag by immunofluorescence as described below (O'Neill and Carroll, 1978; O'Neill *et al*, 1982). The infectious titer was expressed as the highest dilution able to produce T-Ag–positive cells.

In a second method, viral DNA was recovered from infected cells, purified, and quantitated by spectrophotometric measurements at 260 and 280 nm (O'Neill *et al*, 1998). Alternately, the viral DNA was subjected to serial 10-fold dilutions and measured by PCR recovery. The highest dilution at which PCR recovered recognizable viral DNA represented the approximate titer of virions. All of these methods, including T-Ag analysis, were found to give consistent results. For instance, both GS/K + Mad-1– and GS/K + GS/B–cotransfected cells always had the highest titer and GS/B the lowest titer. This occurred in all the assays and the titers for GS/K + Mad-1– and GS/K + GS/B–coinfected cells was equivalent to 1×10^7, whereas GS/B was 1×10^3 to 1×10^4.

Coinfection of cells
HFB cells were cotransfected with GS/K plus GS/B or GS/K plus Mad-1 DNAs and maintained for 1 to 5 weeks. Lysates of these cultures were serially passed to naïve cells when CPE was extensive or, if CPE was not observed, at 1 to 5 weeks after CPE would have been expected to appear.

Immunofluorescence assay of JCV-infected cultures
Infected cells grown on 22-mm^2 cover slip were fixed in a 95% solution of 95% acetone/5% ethanol for 20 s at 20°C, incubated for 30 min at 37°C with a 1:100 dilution of monoclonal mouse anti-Tag (PA6 416), or anti-Vp1 (PAb 597) immunoglobulin G (IgG), washed in phosphate-buffered saline (PBS), and labeled for 25 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody, as previously described (O'Neill and Carroll, 1978; O'Neill *et al*, 1982). Both monoclonal antibodies were originally produced against SV40 T-Ag and Vp1 but shown to react with JCV proteins. Cultures were then examined using a Nikon confocal fluorescence microscope.

PCR analysis of viral DNA
Viral DNA was obtained from infected cultures by Hirt extraction. PCR analysis of DNAs from infected cells was carried out using primers for the JCV regulatory region, JRR 25 and JRR D (Agostini *et al*, 1997b; Elsner and Dörries, 1992). JRR 25: nucleotides 5981 to 5013, CAT GGA TTC CTC CCT ATT CAG CA. JRR D: nucleotides 542 to 521, GCA AGT GCT GCA CCC ATG AAC C. The primers were used at a concentration of 50 pmoles and the DNA was subjected to PCR amplification in a Perkin Elmer Cetus DNA thermal cycler. Cycling was done at: 94°C/3 min once and then 94°C/1 min, 59°C/1 min, 72°C/1 min for 35 to 40 cycles by methods described previously (White *et al*, 1992; O'Neill *et al*, 2003).

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