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BK virus has tropism for human salivary gland cells in vitro: Implications for transmission

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ABSTRACT

Background: In this study, it was determined that BKV is shed in saliva and an in vitro model system was developed whereby BKV can productively infect both submandibular (HSG) and parotid (HSY) salivary gland cell lines.
Results: BKV was detected in oral fluids using quantitative real-time PCR (QRTPCR). BKV infection was determined using quantitative RT-PCR, immunofluorescence and immunoblotting assays. The infectivity of BKV was inhibited by pre-incubation of the virus with gangliosides that saturated the major capsid protein, VP1, halting receptor mediated BKV entry into salivary gland cells. Examination of infected cultures by transmission electron microscopy revealed 45–50 nm BK virions clearly visible within the cells. Subsequent to infection, encapsidated BK virus was detected in the supernatant.
Conclusion: We thus demonstrated that BKV was detected in oral fluids and that BK infection and replication occur in vitro in salivary gland cells. These data collectively suggest the potential for BKV oral route of transmission and oral pathogenesis.

Background

BK virus (BKV) belongs to the polyomavirus family and is ubiquitous in the human population (Fields et al., 2007). The viral capsid is icosahedral and has a diameter of 45–50 nm. The genome consists of a closed circular double-stranded DNA molecule with approximately 5 kb. BKV genome consists of an early region, a late region and a regulatory region (RR) containing promoters, enhancers and the replication origin. The genome is transcribed bi-directionally from the origin, with the early region encoding for two non-structural proteins: large tumor antigen (T Ag), and small tumor antigen (t Ag). The late region encodes for structural capsid proteins VP1, VP2 and VP3 and the non-structural agnoprotein (Fields et al., 2007). BKV initially binds to its cell surface receptor, an N-linked glycoprotein or ganglioside GD1b or GT1b, all of which contain an α(2,3)-linked sialic acid residue (Dugan et al., 2007; Low et al., 2006) and is caveolae-mediated endocytosed and traffics to the nucleus via the endoplasmic reticulum (Dugan et al., 2006). Viral uncoating is believed to occur within tubulo-reticular structures at the nuclear periphery prior to nuclear entry (Drachenberg et al., 2003; Dugan et al., 2006). Once in the cell nucleus, the uncoated chromosome is transcribed. Early gene transcription products result in T antigens that cause quiescent cells to re-enter the cell cycle and increase cellular growth and proliferation. In permissive host cells the T antigens, acting as regulatory proteins, direct the remaining events, resulting in a productive infection. The completion of the process consists of viral DNA replication and transcription of late genes for the production of the structural proteins (VP1, VP2 and VP3) that constitute the capsid (Dugan et al., 2006; Eash et al., 2004; Fields et al., 2007; Hirsch and Steiger, 2003; Low et al., 2004). Viral capsomeres assemble around the daughter chromosomes in the nucleus, to form stable viral particles. Viral egress is thought to occur via cell lysis, although virions have been detected within vesicles in the cytoplasm (Drachenberg et al., 2003).

BKV infection typically occurs during childhood, without specific symptoms, followed by a state of non-replicative infection in various tissues, with the urogenital tract as the principal site (Hirsch, 2005). In the setting of relative or absolute cell-mediated immunosuppression, dramatic increase in BK viral replication occurs, resulting in the lytic destruction of infected uroepithelial cells, which in turn induces the influx of inflammatory immune cells (Hirsch, 2002). This destruction of kidney cells most often occurs in 5–8% of kidney transplants resulting in organ loss in half of these cases and is termed BKV-associated nephropathy (BKVN) (Dugan et al., 2006; Hirsch and Steiger, 2003; Nickeleit et al., 2000). Rise in the incidence of Polyomavirus viruria and viremia has been detected in recipients of bone marrow, kidney and heart transplants, as well as an increase in viruria in HIV-infected individuals (Eash et al., 2006). The potential for

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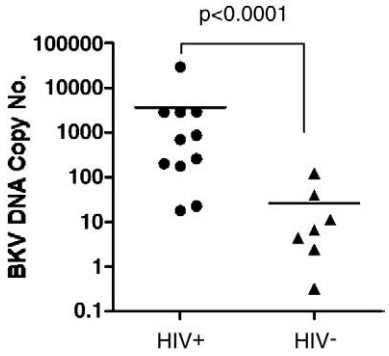

Fig. 1. BKV DNA detection in oral fluids. BKV DNA levels in the saliva were measured by real-time PCR and were found to be significantly higher in patients diagnosed with HIV-associated salivary gland disease compared to patients without the disease. BKV levels in persons with HIV-SGD ranged from 10^1 to 10^4 copies/ml while non-diseased persons ranged from 10^{-2} to 10^2 copies/ml. $p<0.0001$.

BK replication at distant sites such as the salivary gland may certainly exist in the setting of HIV infection (Eash et al., 2006; Webster-Cyriaque, personal communication).

While it is clear that BKV infection is an ubiquitous childhood infection, BKV transmission is not currently well understood. The present studies were undertaken to determine whether BKV could infect and replicate within salivary gland cells resulting in virus production and potentially transmission. We have detected BKV in the saliva of patients with HIV-associated salivary gland pathology and in healthy individuals. In order to begin to decipher BKV pathogenesis within the salivary gland cell it was essential to develop an in vitro model system. We have chosen to characterize BKV infection in submandibular (HSG) and parotid (HSY) gland cell lines. These cells were able to support viral entry, transcription, translation and virion production and BKV infection could be inhibited by saturating the capsid protein with its ganglioside receptor.

Results

BKV DNA detection in oral fluids

Salivary shedding of BKV was assessed in a small cohort of healthy and immune suppressed individuals who had HIV disease. The mean BK copy number in saliva from individuals with HIV and an associated salivary gland disease was 3644 ($n=11$). HIV negative individuals had mean BK copy numbers that were significantly less at 26 ($n=7$) (Fig. 1).

BK virions enter human salivary gland cells

To determine whether BKV could enter salivary gland cells in vitro virion labeling was employed (Fig. 2). Fig. 2 shows CsCl purified BKV labeled with Alexa-Fluor™ 488 (green) and the stained nucleus (red). Bovine serum albumin (BSA) and 1× PBS (data not shown) were also labeled with Alexa-Fluor™ 488 as positive and negative labeling controls, respectively. HSG or Vero cells were incubated with BKV at 64 HAU for 24 h, washed and fixed. Using confocal visualization, at 24 h BKV particles were located in the cell cytoplasm of both HSG and Vero cells. At 48 h BKV accumulation was detected at the perinuclear region of HSG cells. This type of perinuclear accumulation has previously been described in human kidney cells in vivo (Drachenberg et al., 2003) and in vitro (Moriyama and Sorokin, 2008). We did not observe perinuclear accumulation of virions in Vero cells at the

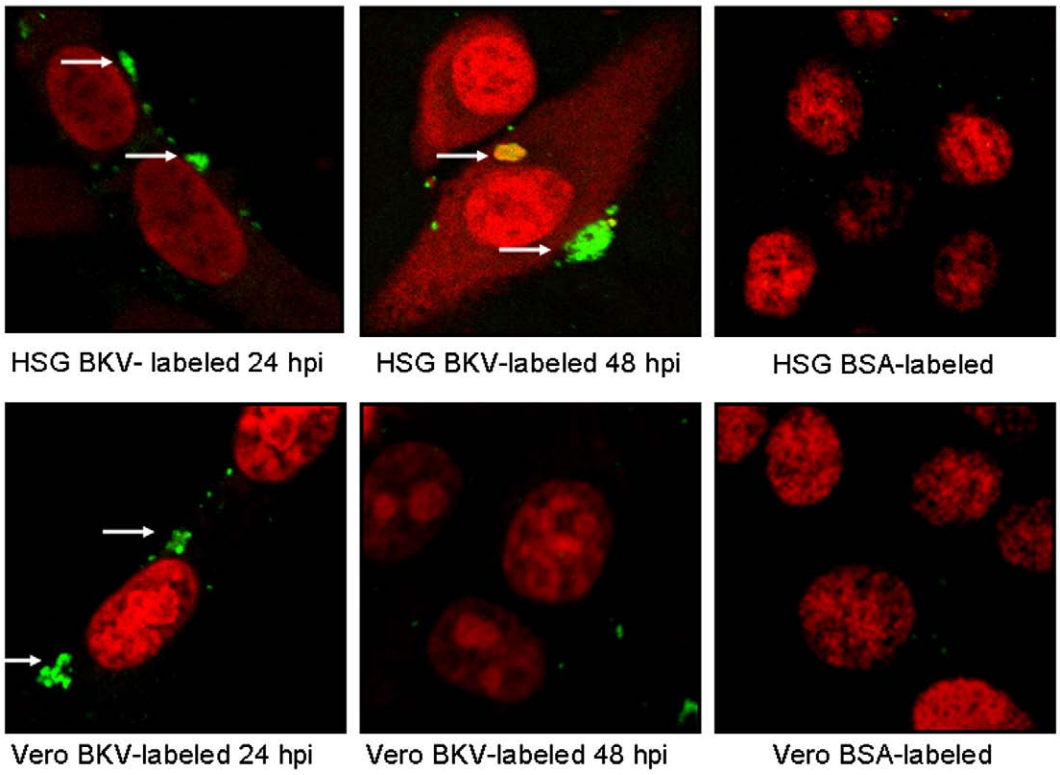

Fig. 2. Detection of BK virions in salivary gland cell lines infected with BKV. (A) HSG and Vero cells were infected with Alexa-Fluor 488-labeled BK virus (green) or Alexa-Fluor 488-labeled BSA (green) or Alexa-Fluor 488-labeled 1X PBS (data not shown) as stated in the Materials and methods. At stated times post-infection, monolayers of cells were fixed and observed by confocal microscopy using a 63× lens objective. At 24 hpi, labeled BKV (green) was detected at the plasma cell membrane of HSG cells and within the cytoplasm of Vero cells. At 48 hpi, labeled BKV (green) was detected at the perinuclear region of HSG cells and not at all detected in Vero cells. Labeled BSA (green) and 1× PBS (data not shown) was detected only in the extracellular regions of both HSG and Vero cells. TO-PRO3 dye was used to stain the cell nuclei (red). White arrows point to Alexa-Fluor 488-labeled BK virions.

48 h time point suggesting that viral uncoating and nuclear entry may have already occurred. It has previously been shown in human kidney cells that BKV traffics from the cell surface to the nucleus within 12 to 24 h (Moriyama and Sorokin, 2008) which may explain our inability to detect labeled BKV at the stated 48 h time point in Vero cells. The kinetics of entry and uncoating may be cell type specific.

BKV transcripts expressed in salivary gland cells

Upon determination of BKV entry into salivary gland cells in vitro, the outcome of infection was assessed. Viral transcription levels of early gene, T Ag and the late gene, VP1 were examined. In both submandibular (HSG) and parotid gland (HSY) cell lines an increase in viral transcription of both T Ag and VP1 genes was observed up to 6 dpi (Fig. 3). Beyond 6 dpi, cells became over-confluent, lifted off of the plate surface and began to die. A steady increase in transcription of both T Ag and VP1 transcripts was detected, generally peaking at 3 dpi. We consistently observed higher levels of VP1 versus T Ag transcripts in both cell types. This is consistent with the requirement for increased amounts of structural protein as compared to non-structural protein for viral replication to occur. Small amounts of T Ag are necessary to activate late gene transcription. Results were normalized to the levels of β-actin, using the $2^{-C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels. Uninfected, mock samples were arbitrarily set to 1.

BKV protein expression in salivary gland cells

Translation of T Ag in BKV-infected HSG cells was assessed by immunoblotting and immunofluorescence techniques (Figs. 4A, B and C). As expected protein expression was delayed compared to mRNA transcription, consistent with previous work showing that BK virus replicates slowly in vitro (Dugan et al., 2006; Low et al., 2004; Moriyama and Sorokin, 2008). Nuclear T Ag and VP1 protein expression was first detected at 4 dpi by IFA (Fig. 4B and 4A). Based on counting of IF positive cells at 5 dpi (data not shown), an average of ten experiments determined the percentage of VP1 positive cells was approximately 15% and the percentage of T ag positive cells were 10% or less. Immunoblot consistently detected expression of both small and large T antigen at 4 dpi, although in one infection small amounts of protein were detected at day 3 (Fig. 4C top and middle panel). Collectively, these data indicate that BKV gene expression occurred in human salivary gland cells in vitro.

Salivary gland cells release infectious virus into the supernatant

In order to determine whether BKV infection of salivary gland cells results in productive infection, release of infectious virions from salivary gland cells was assessed. BK virions were not detected by electron microscopy in salivary gland cells days 2–4 dpi. However, at 5 dpi 45–50 nm BK virions were identified within the cell cytoplasm similar to those virions detected within BKV-infected kidney cells (decoy cells) shed into the urine of a kidney transplant patient diagnosed with BKV nephropathy (Fig. 5). No viral particles were detected prior to 5 dpi within the cell cytoplasm.

Time courses were performed during which supernatants from HSG cells were collected and filtered at 0, 1, 3, 4 and 5 days post-infection. Two separate experiments were performed in which 1) separate plates were infected and harvested at stated time points (data not shown) or 2) a single plate was infected from which supernatant was collected over time (Fig. 6). Supernatants were then DNAse/Proteinase K treated to detect only encapsidated viral DNA

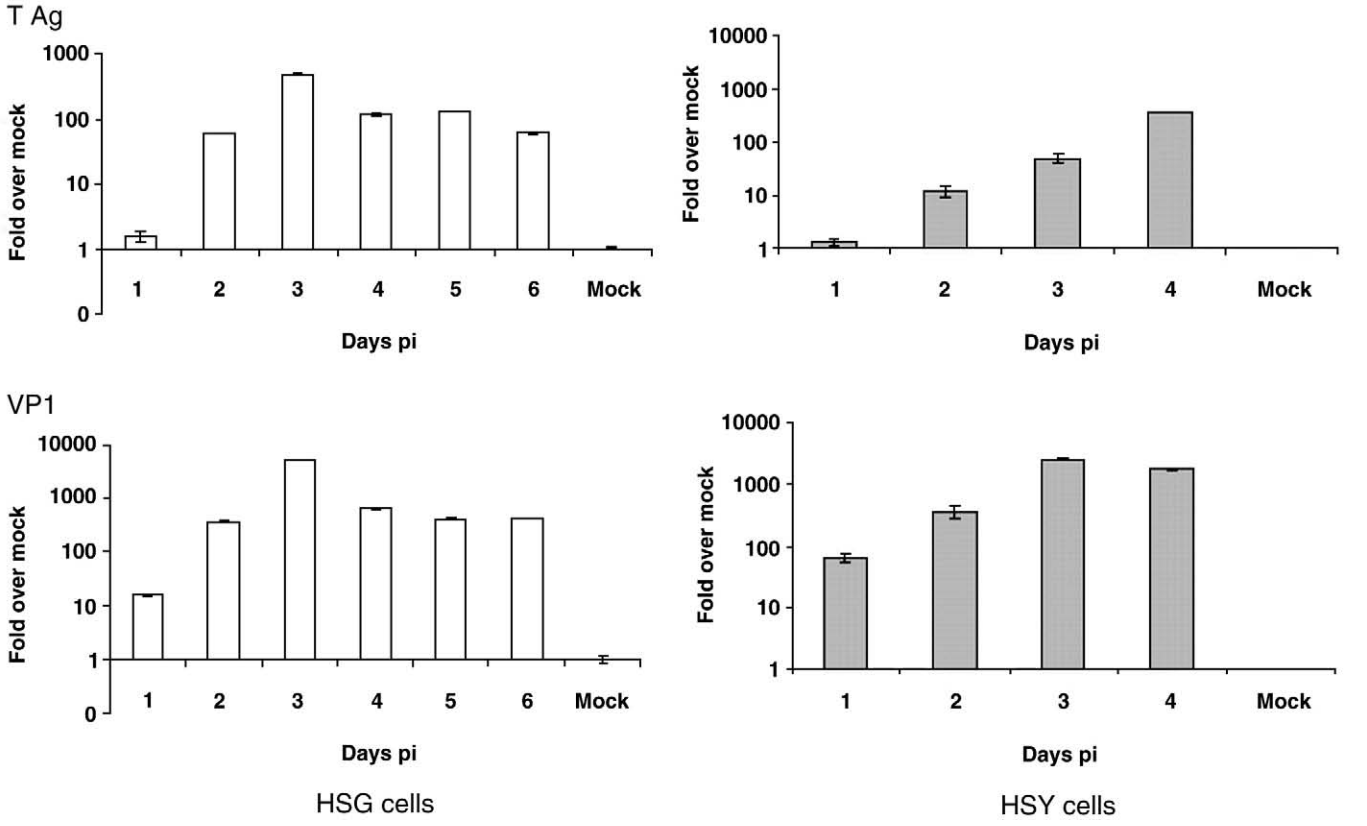

Fig. 3. BKV replicates in human salivary gland cells. HSG and HSY cells were infected with BKV or media alone (mock) as described in the Materials and methods. (A) At stated times post-infection cells were collected, RNA isolated, cDNA generated and quantitative real-time PCR performed for T Ag and VP1 viral transcripts. Gene expression values were normalized to the levels of β-actin transcripts, using the $2^{-C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set to 1.

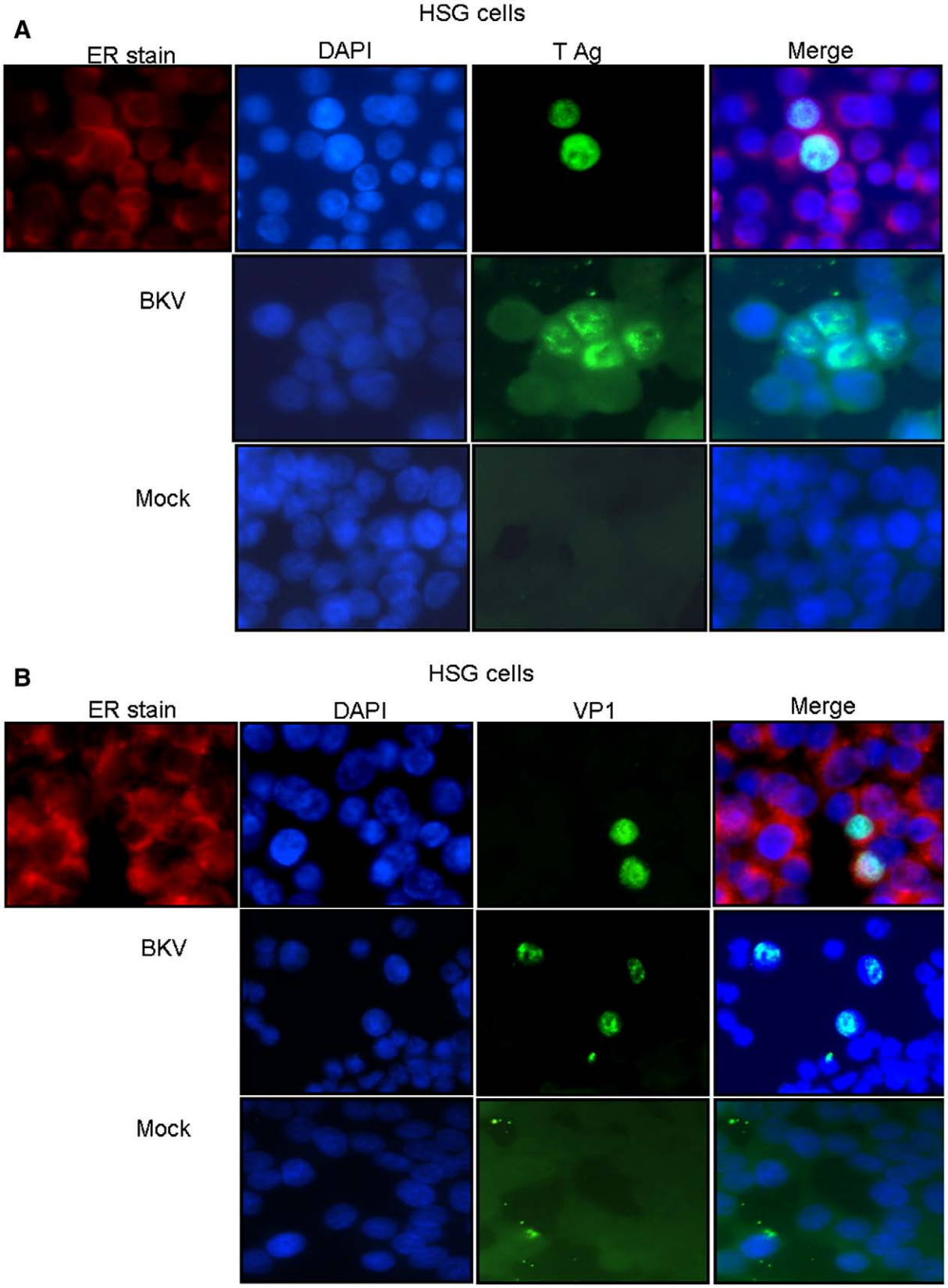

Fig. 4. At 4 dpi, monolayer of cells were fixed and probed for (A) T Ag and (B) VP1 expression using PAb 416 and NCL-JCBK antibodies, respectively. Photos are representative BKV-infected (top and middle panel) or control mock (bottom panel) HSG cells stained for T Ag (green) and VP1 (green). DAPI (blue) was used to stain the cell nuclei and Brefeldin A (red) used to stain the endoplasmic reticulum. (×20 magnification). (C) At stated times post-infection cell lysates were collected and used for immunoblotting as described in the Materials and methods. Antibodies against T Ag (top panel) and β-actin (bottom panel) were used. T Ag protein was first detected at 4 dpi.

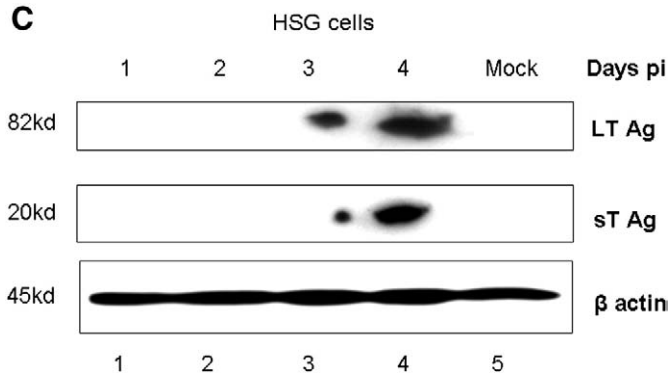

Fig. 4 (*continued*).

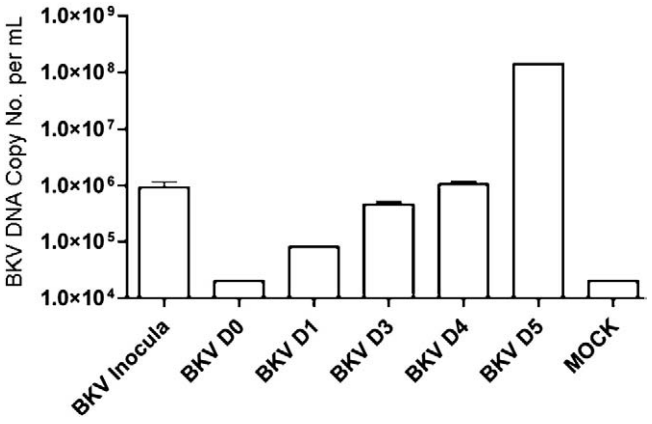

Fig. 6. Encapsidated BK virions released from human salivary gland cells. Supernatant of BKV or mock (media only) infected HSG cells was collected at 0, 1, 3, 4 and 5 dpi, filtered and treated with either proteinase K only to quantify total vDNA (data not shown) or DNase followed by proteinase K treatment, to quantify encapsidated vDNA only, as described in the Materials and methods. Eluted vDNA was then used to quantify T Ag copy number using real-time PCR analysis. A standard curve (data not shown) was constructed using a plasmid coding for BKV T Ag to determine copy number.

(vDNA) or Proteinase K-only treated as a control to detect both encapsidated and unencapsidated vDNA (data not shown), as previously described (Dugan et al., 2007). Over time post-infection increasing levels of virus were detected by quantitative real-time PCR indicating release of encapsidated viral DNA from infected cells.

The supernatants from HSG cells were also used to infect naive Vero cells. At 5 days post Vero cell treatment, cells were collected for quantitative real-time PCR, immunofluorescence, and immunoblotting. Again, real-time results were normalized to the levels of β-actin transcripts using the $2^{-C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels, with the levels in mock-treated samples arbitrarily set to 1. The level of T Ag and VP1 viral transcripts, was significantly higher in Vero cells treated with BKV-infected HSG supernatant over mock-infected HSG supernatant (Fig. 7A). Immunofluorescence and immunoblotting techniques detected T Ag protein expressed in Vero cells treated with BKV-infected HSG supernatant (Figs. 7B and C). These data suggest that human salivary gland cells can indeed be productively infected with BK virus.

BKV infects salivary gland cells via gangliosides

It has previously been shown that BKV's receptors include an N-linked glycoprotein with α(2,3)-linked sialic acid and gangliosides, GD1b and GT1b (Dugan et al., 2005; Low et al., 2006). As HSG cells

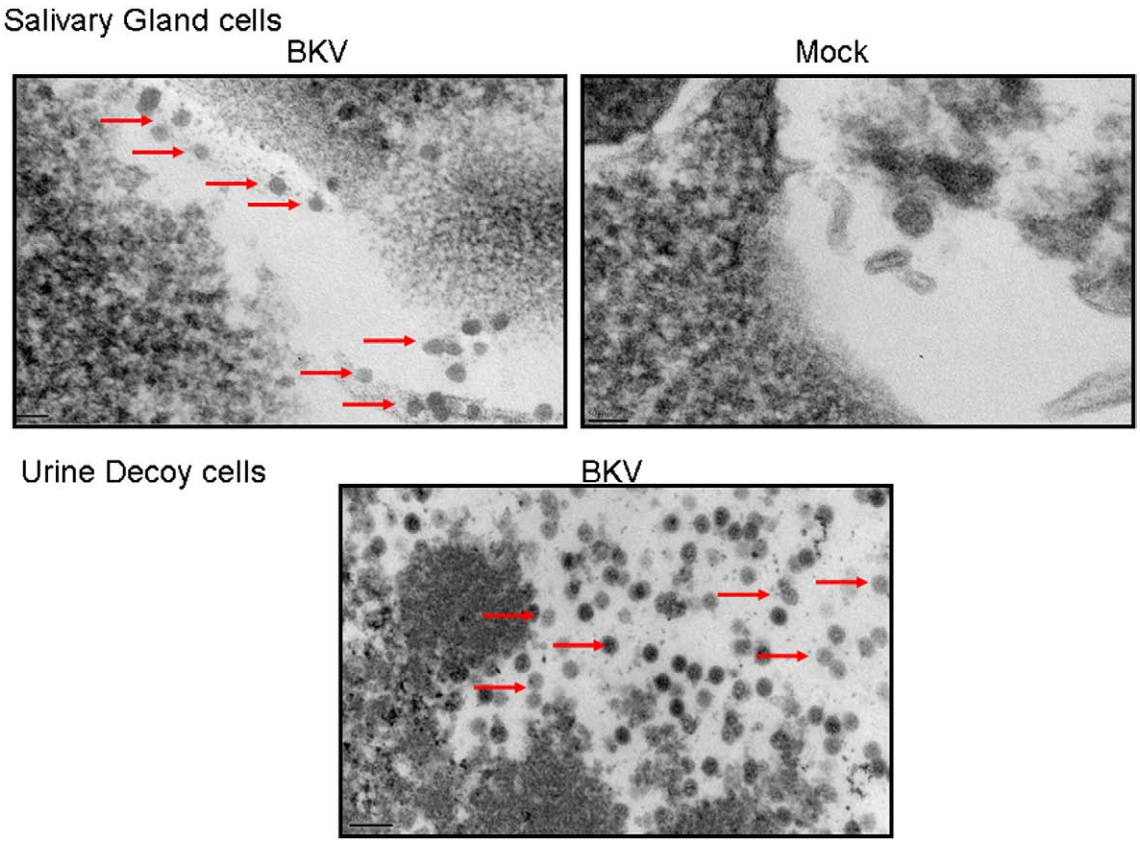

Fig. 5. Representative transmission electron micrograph (TEM) of BK virions at ×160,000 magnification of BKV and mock-infected HSG cells (top panel) at 5 dpi. Bottom panel, BK virions from BKV-infected kidney cells (decoy cells) in the urine of a BKV-associated nephritis patient.

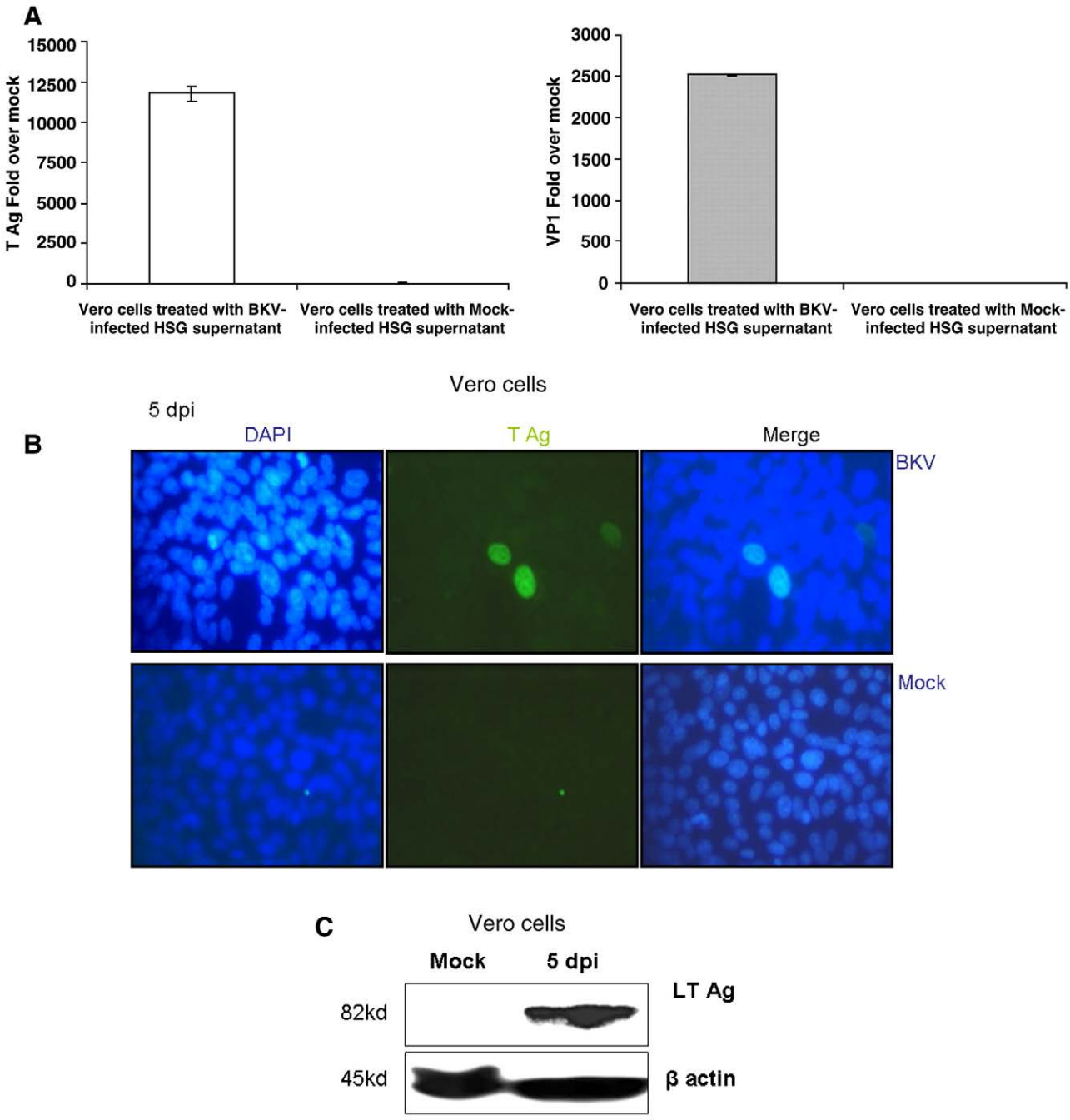

Fig. 7. Infectious virus released from HSG cells. Supernatant of BKV or media only (mock) infected HSG cells was collected at 5 dpi, filtered and used to infect Vero cells as described in the Materials and methods. At 5 days post treatment with HSG supernatant, Vero cells are examined for BKV infection by (A) quantitative real-time PCR analysis (B) immunofluorescence and (C) immunoblot. (A) For real-time PCR analysis, gene expression values were normalized to the levels of β-actin transcripts, using the $2^{-C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set to 1. (B) Vero cells were fixed at day 5 dpi and stained for T Ag (green). DAPI (blue) was used to stain the cell nuclei. Representative photograph of BKV T Ag and nuclear staining are shown for BKV-infected and control mock Vero cells. (C) Immunoblot analysis of Vero cells for BKV T Ag (upper panel) and β-actin (lower panel) proteins detected at 5 days post HSG supernatant treatment.

were permissive for BKV infection, the use of gangliosides for receptor-mediated BKV entry into these cells was assessed. A mixture containing all of these gangliosides in addition to GM1 and GD3 was utilized as the receptor for BKV on salivary gland cells was unknown. BKV was pre-incubated with GM1, GD1b, GT1b and GD3 to bind and saturate the capsid protein, VP1. Two different concentrations of ganglioside: 60 μg/ml and 100 μg/ml, were pre-incubated with BK virus prior to infection of HSG and Vero cells. Using real-time quantitative PCR, a 28-fold decrease was detected in viral transcription of T Ag in HSG cells infected with both BKV/60 μg and BKV/100 μg ganglioside mix compared to cells infected with BKV/DMSO alone. A 273-fold and 72-fold decrease was detected in Vero cells infected with BKV/60 μg and BKV/100 μg, respectively (Fig. 8A). For VP1 viral transcripts in HSG cells we observed a 46 and 81-fold decrease in BKV/60 μg and BKV/100 μg ganglioside mix, respectively, compared to cells infected with BKV/DMSO alone. In Vero cells we detected a 402 and 62-fold decrease in VP1 transcripts when infected with BKV/60 μg and BKV/100 μg ganglioside mix, respectively.

A decrease in the number of T Ag expressing cells was also detected by IFA upon infection with the BKV/ganglioside mix versus cells exposed to BKV/DMSO (Fig. 8B). In HSG cells, at 4 dpi, a 75% decrease in T Ag expressing cells was detected with the BKV/100 μg gangliosideinfection (data not shown) and a 45% decrease detected with BKV/60 μg ganglioside infection (Fig. 8B). While at 6 dpi, we observed 61% and 96% percent decrease in BKV/60 μg and BKV/100 μg infections, respectively (Data not shown). In Vero cells, at 4 (Fig. 8C) and 6 dpi (Data not shown), a100% decrease was detected with both the BKV/60 μg and BKV/100 μg ganglioside infections. These data indicate that human salivary gland cells are infected with BKV via receptor-mediated VP1 entry and suggest that there may be a difference in receptor abundance between salivary gland cells and kidney cells.

Discussion

The present data demonstrate for the first time, BKV detection in saliva and evidence that human salivary gland cells can be productively infected with BK virus. This suggests that while kidney/uroepithelial cells have long been known to be a site of BKV replication and latency, the salivary gland may also constitute an infectious reservoir for BKV. Although the natural route of BKV transmission has not been resolved (Fields et al., 2007; Hirsch, 2005), our studies suggest a potential for oral BKV transmission.

Previous studies have investigated the presence of BKV DNA by polymerase chain reaction (PCR) in saliva from HIV-immunodeficient individuals and healthy controls but were not detected in their cohort (Sundsfjord et al., 1994). Our studies however, using quantitative real-time PCR analysis detected BKV DNA in saliva samples from HIV positive patients and healthy controls. And like most opportunistic pathogens, BKV DNA was detected in the saliva at higher levels in immunocompromised patients compared to healthy individuals.

The detection of labeled BK virions within the cytoplasm of salivary gland cells confirmed BKV entry. In salivary gland cells, labeled virus was still detected at 48 hpi while labeled virus was no longer detected in Vero cells at the same time point perhaps due to viral uncoating and entry into the nucleus. Viral gene products were first observed at 24 hpi for both T Ag and VP1 using real-time RT-PCR, with viral transcription peaking at about 3 dpi for both submandibular and parotid gland cell infection. Protein expression was first detected at 4 dpi by immunoblot and IFA. While BKV transcripts were detected in abundance the expression of viral encoded proteins was more modest. The difference in mRNA versus protein levels may reflect the decreased stability of the BKV T Ag protein. Productive infection was further substantiated by the detection of virions by EM at 5 days post-infection, as well as the presence of DNAse resistant virus in the supernatant. These virions increased over time and were used to infect naïve Vero cells. Interestingly, the kinetics of infection appeared to be slower in salivary gland cells than in kidney cells.

BKV entry into salivary gland cells was inhibited by blocking VP1 via ganglioside saturation, as has previously been shown in Vero cells (Sinibaldi et al., 1990). As expected viral transcription was more significantly diminished in the presence of 100 μg/ml ganglioside compared to 60 μg/ml in the salivary gland cells. Similar trends were observed in the Vero cell line upon ganglioside treatment. Likewise a consistent decrease in viral protein expression was detected with ganglioside treatment in both salivary gland and Vero cells.

The high incidence of BKV infection in the world population raises obvious questions about the mode of transmission from one individual to another. Given the known latency of the virus in the kidney, urine would appear to be a natural vehicle for spread within and between families. However, a variety of laboratory techniques have been used to assess the prevalence of viruria in the pediatric age groups, resulting in very low yield varying from 4% to 26.7% (Knowles, 2001). Transplacental transmission of BKV from mother to fetus has also been evaluated (Coleman et al., 1980; Pietropaolo et al., 1998; Shah et al., 1980; Taguchi et al., 1975). The data thus far however is controversial, with one group demonstrating 50% of their infants expressing BKV-specific IgM antibodies in infants whose mothers seroconverted during pregnancy (Pietropaolo et al., 1998; Taguchi et al., 1975). While others have shown consistently negative results for BKV-specific IgM in neonatal and cord blood samples drawn from the offspring of mothers excreting viral inclusion bearing cells in the urine (Shah et al., 1980). Transplacental transmission of polyomavirus has been demonstrated in mice (McCance and Mims, 1977), and it is conceivable that the same could occur in man. BKV DNA has also been detected in feces, blood, semen, genital tissues, peripheral blood mononuclear cells (PBMC), and normal skin biopsies, hence it is possible that the virus maybe transmitted by intimate contact with infected individuals (Bofill-Mas et al., 2000; Chatterjee et al., 2000; Monini et al., 1995).

The possibility of feco-oral transmission has recently been raised upon the detection of BKV DNA in urban sewage (Bofill-Mas et al., 2000). This possibility may also be considered as potentially complementary to our studies because swallowed material passes the mouth and nasopharynx and viruses transmitted through the oral–fecal route may be able to multiply in the throat, for example, in the salivary glands or tonsils. Additionally, both BKV and SV40 have been detected in the tonsils of immunocompetent children (Patel et al., 2008) and BKV detected in the nasopharyngeal aspirates of children with acute respiratory tract disease (Goudsmit et al., 1982; Patel et al., 2008; Sundsfjord et al., 1994). Again, consistent with our studies indicating that BKV is present in the oral cavity and is possibly a mode of transmission from human to human. The presence of BKV in the saliva and its ability to replicate in salivary glands cells in vitro further suggests a role for oral transmission since BKV was detected in the saliva of both healthy and diseased patients in our study.

Conclusion

In conclusion, we have detected BKV in oral fluids and have shown that BKV is capable of permissive infection of salivary gland cells in vitro. To our knowledge these studies provide the first analysis of BKV salivary gland infection and replication. To the extent that parotid (HSY) and submandibular gland (HSG) cells can sustain low levels of BKV replication, substantiates BKV's presence in oral fluids originating within these glandular cells and confirms the potential for BKV oral transmission. These data may provide a foundation critical for further understanding BKV infection and pathogenesis. The experiments described in this paper define a system in which BKV lytic replication can be studied in vitro using a physiologically relevant cell type. The characterization of BKV infection of these cells sets the groundwork for future studies to better understand how BKV is able to persist within the human host and reactivate under immuno-suppressed conditions. Further studies will help us to identify factors that influence the vulnerability of salivary gland epithelial cells to BKV infection and more fully define BKV's role in disease. Determination of the cellular pathways that are altered may allow us to target and implement appropriate interventions that would decrease the morbidity associated with BKV-associated salivary gland pathologies.

Materials and methods

Patients and sample collection

HIV positive patients from UNC hospital's dental clinic/infectious disease clinic and healthy volunteers were recruited to participate in the IRB approved study (04-DENT-356). Five-milliliter saliva samples from HIV positive and negative individuals were collected, contents centrifuged and pelleted and DNA extracted by Qiagen DNeasy kit according to the manufacturers' instruction.

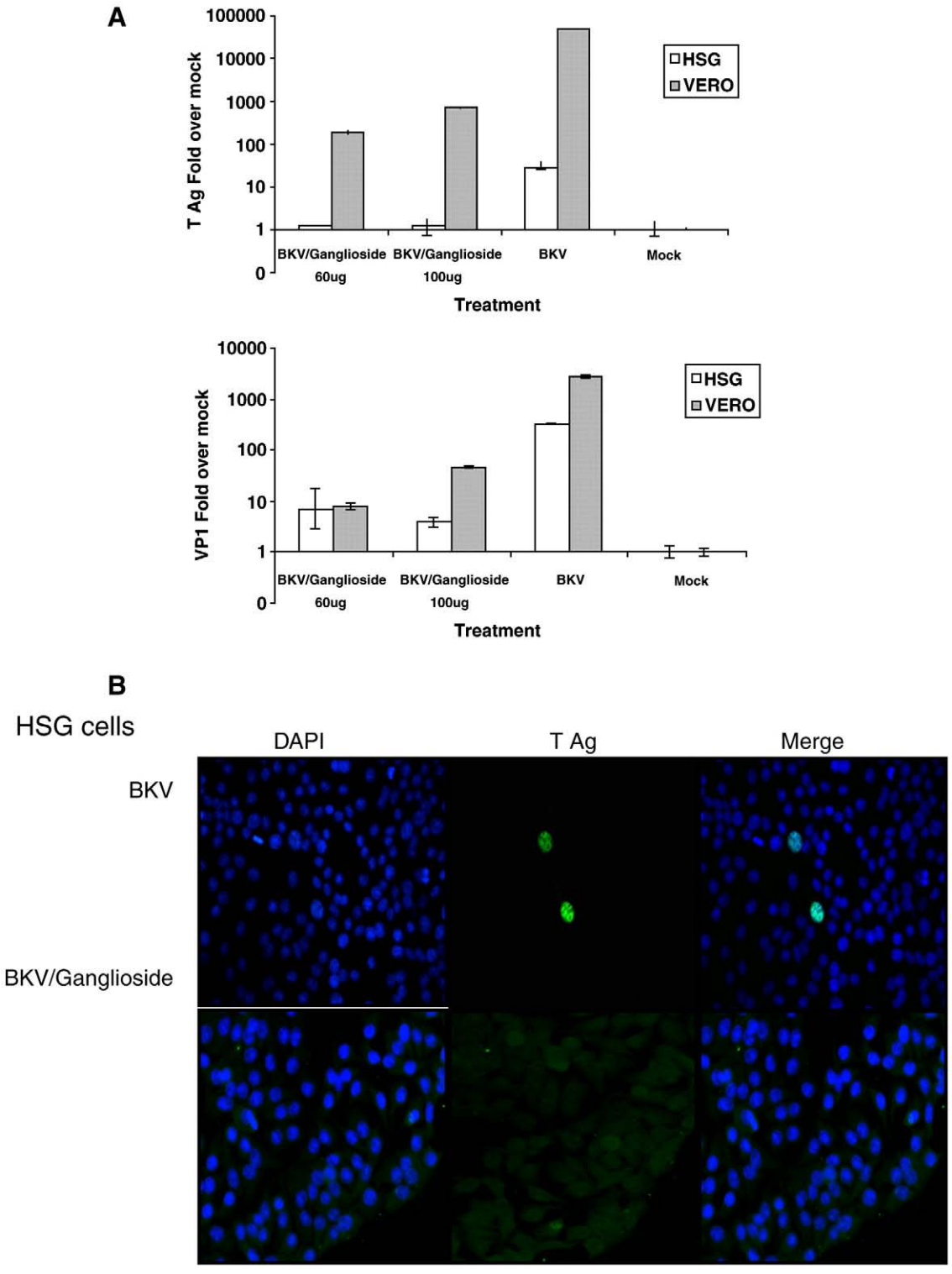

Fig. 8. Inhibition of BKV infection in salivary gland cell lines via gangliosides. HSG and Vero cells were infected with BKV in the absence or presence of a ganglioside mix or negative control media only (mock) and analyzed for T Ag expression at stated times post-infection. (A) Real-time RT-PCR analysis of T Ag (top panel) and VP1 (bottom panel) transcripts from HSG (white bar) and Vero (checkered bar) cells at 4 dpi. Two concentrations of ganglioside mixtures were used, 60 μg/ml or 100 μg/ml, to incubate with BKV prior to infection. Gene expression values were normalized to the levels of β-actin transcripts, using the $2^{-C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag/VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set to 1. Shown is a representative experiment, this study was performed three times in duplicate. (B) Immunofluorescence analysis of T Ag protein expression from HSG (top panel) and (C) Vero (bottom panel) cells. At 4 and 6 dpi (data not shown) monolayer of cells were fixed and stained for T Ag (green) and DAPI (blue) used to stain the cell nuclei representative photos of IFA staining for T Ag in HSG and Vero cells at 4 dpi treated with 60 ug/ml ganglioside mix. (×20 magnification).

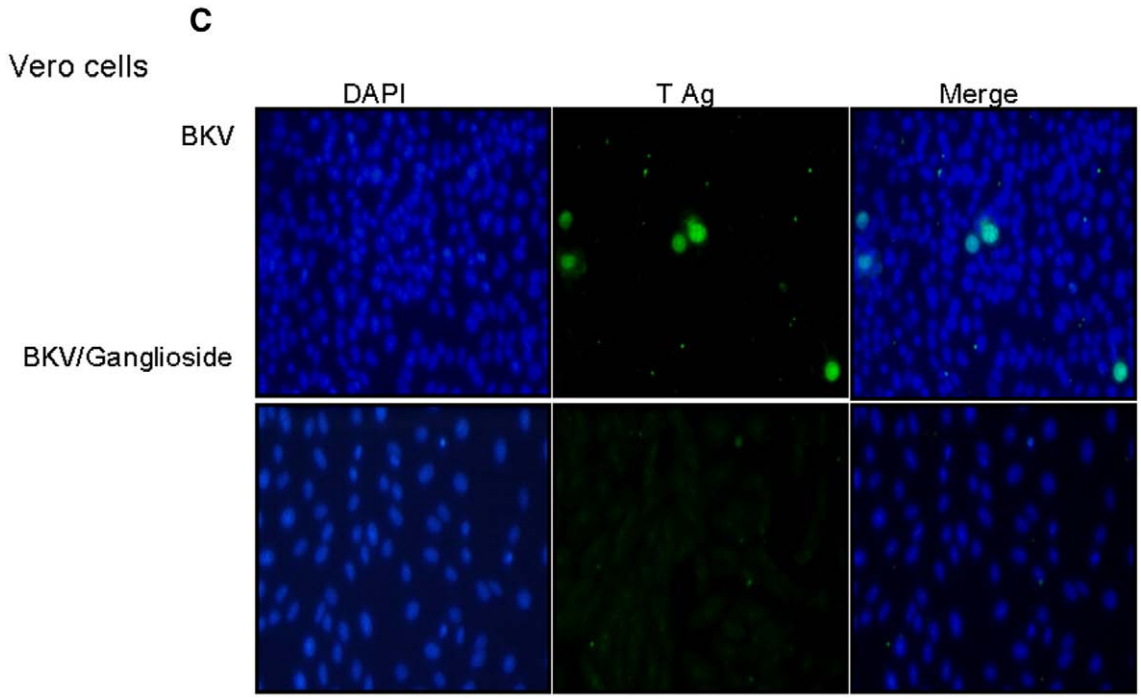

Fig. 8 (*continued*).

Cell culture and virus

HSY cells are a neoplastic epithelial cell line initially established in culture from a human parotid gland adenocarcinoma (Hayashi et al., 1987). HSG cells are an epithelial cell line isolated by using tissue culture techniques from an irradiated human submandibular salivary gland which showed no neoplastic lesion (Shirasuna et al., 1981). HSG and HSY cells were obtained as a gift from Dr. B. Baum (NIH) and cultured in McCoy's 5A medium (Sigma) and Dulbecco's minimal essential medium (DMEM; Sigma), respectively. African monkey kidney cells or Vero cells (American Type Culture Collection [ATCC]) were also cultured in DMEM (Sigma). All cell types were grown in medium supplemented with 10% fetal bovine serum (FBS) (Sigma), and 1% penicillin–streptomycin (pen/strep)(Gibco) unless otherwise stated and maintained in a humidified 37 °C, 5%CO_2 chamber. BKV stocks were initially propagated in Vero cells from virus obtained from ATCC (VR-837). Viral lysates were made through three cycles of freezing the infected cells and supernatant at −80 °C and thawing at 37 °C.

Hemagglutination assay

BK virus was titered using hemagglutination assay as previously described (Raptis, 2001). Briefly, 90 µl of culture fluid was added to 10 µl of 2.5% NP-40 in PBS and incubated at 37 °C for 30 min. Two-fold dilutions of the treated-fluid in 25 µl volume of HA buffer was transferred into V-bottomed 96-well microtiter plates and 25 µl of a cold 0.5% suspension of guinea pig erythrocytes was added to each well. Plates were then incubated at 4 °C for non-agglutinated cells to settle (about 2 h). BK viral titer was taken to be the highest dilution in which >75% of the erythrocytes is agglutinated.

Conditions of infection

HSY, HSG and Vero cell monolayers were trypsinized, washed and resuspended in fresh culture medium supplemented with 2% FBS and 1% pen/strep and plated in poly-L Lysine coated 8-well chamber slides (Falcon), 12 well dishes or 35-mm diameter well culture plates at a concentration of 6.5×10^5 cells/ml. 64 HAUs of BKV was then added to the dish and allowed to infect for 24 h. At 24 h post-infection (hpi), virus was removed from the culture medium, cells were washed with 1× PBS and replaced with fresh medium. The infection of HSG and Vero cells was blocked by pre-incubation of BKV with 60 µg/ml or 100 µg/ml purified mixed gangliosides (Matreya, LLC) in media for 2–6 h at 37° C. At various times post-infection the cell monolayers or supernatant were further processed for IFA, protein, RNA or DNA isolation, as described below.

Indirect immunofluorescence (IFA)

At stated times post-infection, cells were fixed for 10 min with 50% methanol/50% acetone, dried at RT then incubated at −20° C overnight for antigen retrieval. Fixed cells were then thawed at RT, rehydrated with 1× PBS/TritonX and incubated with PAb416 (Genetex) antibody (1:30) specific for SV40T antigen. PAb416 has been shown to cross react with BKV T antigen and is commonly used for BKV T Ag detection. NCL-JCBK (Novocastra) antibody (1:20) was used to detect BKV VP1 protein. NCL-JCBK is specific for both BK and JC VP1 protein detection. Both antibodies were incubated with fixed cells for 1 h at 37° C followed by a fluorescein-conjugated anti-mouse (Sigma) antibody (1:20). DAPI (Invitrogen) (1:10,000) was used to stain the nucleus and brefeldin A, BODIPY 558/568 conjugate to stain the endoplasmic reticulum (Invitrogen). At least ten random fields of positively stained cells were counted to determine percent infection. Nikon FXA with Q camera or Olympus IMT2 fluorescent microscopes were used to take photographs of cells.

Immunoblotting

Total cell protein was extracted using 1% SDS lysis buffer (1% (w/v) SDS, 0.05 M Tris Cl pH8, 1 mM DTT). Protein concentrations were determined using the BioRad protein assay, and equal amounts of protein were electrophoresed on a 10% Bis–Tris polyacrylamide minigel (Invitrogen). PAb416 (1:200) (Genetex) in 5% non-fat dry

milk in 0.1% Tween-20 PBS (PBS-T) was used to detect T Ag expression and Actin (C-11)-R sc-1615-R (1:1000)(Santa Cruz Biotechnology) in 1% BSA/TBS-T for actin expression. After washing in PBS/TBS-T, blots were probed with a horseradish peroxidase-conjugated secondary antibody (1:10,000) (Promega). Antibody complexes were detected using SuperSignal West Pico Chemiluminescent substrate (Thermo scientific) and exposed to film (Kodak).

RNA isolation and real-time RT-PCR amplification

Total RNA was extracted using TRizol (Invitrogen) as described by the manufacturer. Contaminating DNAs were removed by use of RQ1 DNase kit (Promega) as described by the manufacturer. cDNA was generated using 20 μg RNA, random primers and the SuperScript™ II Reverse Transcriptase (RT) Kit (Invitrogen) as described by the manufacturer. A non-RT enzyme reaction was performed for each sample as a negative control for cDNA synthesis. cDNA was then subjected to real-time PCR analysis using Roche LightCycler 480 Syber Green I Master Mix as a detector in the Roche Light Cycler 480. Previously published primers for T Ag (Dana et al., 2005) and VP1 (Ding et al., 2002) were used. Gene expression values were normalized to the levels of β-actin transcripts, using the $2^{-C(T)}$ method, and are presented as the changes (*n*-fold) in T Ag and VP1 transcript levels, with the levels in uninfected/mock samples arbitrarily set to 1.

Encapsidated viral genome assay

The detection of infectious virus being released from HSG cells was performed on HSG cell supernatant collected at stated times post-infection. Supernatant was passed through a 0.45 μM filter and DNase-treated or used to infect Vero cells as previously described (Dugan et al., 2007). To degrade free DNA not encapsidated within virions, supernatant was treated with 250 U DNase (Promega) or water for 15 min at 56 °C, followed by enzyme inactivation at 65 °C for 10 min. To release viral DNA (vDNA) from capsids, proteinase K was used as described in the blood and body fluid spin protocol of the QIAamp DNA blood minikit (QIAGEN). DNA was eluted in 100 μl of sterile water. Levels of viral DNA were determined using primers for T Ag in real-time PCR as described above. A plasmid, pBKV containing the entire genome of BKV, a gift from Volker Nickeleit (UNC-CH) was used to derive standard curves for viral DNA quantitation.

Virus purification and labeling and confocal microscopy

BKV was propagated in Vero cells and purified as previously described (Low et al., 2006). Briefly, viral lysates, including all cell debris, were adjusted to pH 7.4 with 0.5 M HEPES (pH 8.0) and centrifuged at 8000 ×*g* for 30 min at 4 °C. The supernatant was removed, and the pellet was resuspended in 5 ml of buffer A (10 mM HEPES [pH 7.9], 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM KCl). The pH of the lysate was then adjusted to 6.0 with 0.5 M HEPES (pH 5.4), and 5 units of type V neuraminidase (Sigma) was added and incubated for 1 h at 25 °C. The solution was returned to pH 7.4 through the addition of 0.5 M HEPES (pH 8.0) and then heated to 40°C and centrifuged at 16,000 ×*g* for 5 min. The supernatant was removed and saved, while the pellet was further incubated with 5 ml of buffer A containing 0.1% deoxycholate at 25°C for 15 min and then centrifuged at 16,000 ×*g* for 5 min. The supernatant was removed, combined with the previous supernatant, and centrifuged through a 15% sucrose cushion into 35 g/100 ml CsCl at 141,000 ×*g* in a Beckman Optima LE-80K ultracentrifuge using a Beckman tube (28 by 89 mm) in a Beckman SW28 rotor for 3.5 h at 20 °C. Virus was dialyzed against buffer A, and titers were determined using the hemmaglutination assay and DNase protection assay.

Purified BKV was then labeled using Alexa-Fluor 488 microscale protein labeling kit (Molecular Probes) as directed by the manufacturer (bovine serum albumin (BSA) and 1× PBS were used as positive and negative labeling controls). Briefly, 100 μl of purified viral solution or 1 mg/ml BSA or 100 μl 1× PBS was transferred to a reaction tube and 10 μl of 1 M sodium bicarbonate added, 33 nmol Alexa-Fluor tetrafluorophenyl ester was added and the reaction mixture was incubated for 15 min. After incubation, 50 μl of the conjugate reaction mixture was layered on the resin bed in the microcentrifuge tube and centrifuged at 16,000 ×*g* for 1 min. The eluted virus was then used to infect HSG or Vero cells in poly-L lysine coated 8-well chamber slides (Falcon). At 24 and 48 hpi, monolayers of cells were fixed with 50% methanol/50% acetone for 10 min followed by addition of TO-PRO3 (Invitrogen) reagent at 1:10,000 for 2 min to stain the cell nucleus. Cells were then washed and stored in 1× PBS and used to detect virion entry using an Olympus FV 500 microscope.

Electron microscopy

HSG cells were infected with BKV or media (mock) and at 5 dpi the cells were washed with 1× PBS and the cell monolayers fixed with 3% glutaraldehyde in 0.15 M sodium phosphate buffer. Cells were then post-fixed with potassium ferrocyanide-reduced osmium tetroxide. Cells were embedded *in situ* in Polybed 812 epoxy resin and cut into ultrathin sections of 70 nm. Sections were post-stained in uranyl acetate and lead citrate then photographed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy Inc. Thornwood, NY) with Orius camera at 80 kV. All sections were cut parallel to the substrate.

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