BK polyomavirus hijacks extracellular vesicles for en bloc transmission.

Lynda Handala, a,b Emmanuelle Blanchard, c,d Pierre-Ivan Raynal, e Philippe Roingeard, c,d Virginie Morel, a,b Véronique Descamps, a,b Sandrine Castelain, a,b Catherine Francois, a,b Gilles Duverlie, a,b Etienne Brochot, a,b/# Francois Helle a,#

a EA4294, Agents Infectieux, Résistance et chimiothérapie (AGIR), Centre Universitaire de Recherche en Santé, Université de Picardie Jules Verne, 80054 Amiens, France.

b Laboratoire de Virologie, Centre Hospitalier Universitaire, 80054 Amiens, France.

c Plateforme IBiSA de Microscopie Electronique, Université de Tours et CHU de Tours, Tours, France.

d INSERM U1259, Université de Tours et CHU de Tours, Tours, France.

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# Corresponding authors:

Dr. F. Helle, EA4294, Agents Infectieux, Résistance et chimiothérapie, Centre Universitaire de Recherche en Santé, Centre Hospitalier Universitaire et Université de Picardie Jules Verne, 80054 Amiens, France.

Phone: (+33)-3-22-82-53-51

E-mail: francois.helle@u-picardie.fr
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ABSTRACT

Most people are asymptomatic carriers of the BK polyomavirus (BKPyV) but the mechanisms of persistence and immune evasion remain poorly understood. Furthermore, BKPyV is responsible for nephropathies in kidney transplant recipients. Unfortunately, the sole therapeutic option is to modulate immunosuppression, which increases the risk of transplant rejection. Using iodixanol density gradients we observed that Vero and renal proximal tubular epithelial infected cells release two populations of infectious particles, one of which co-sedimenting with extracellular vesicles (EVs). Electron microscopy confirmed that a single vesicle could traffic tens of viral particles. In contrast to naked virions, the EV-associated particles (eBKPyV) were not able to agglutinate red blood cells and did not use cell surface sialylated glycans as attachment factor demonstrating that different entry pathways were involved for each type of infectious particles. However, we also observed that naked BKPyV and eBKPyV were equally sensitive to neutralization by the serum of a seropositive patient or commercially available polyvalent immunoglobulin preparations, which occurred at a post-attachment step, after endocytosis. In conclusion, our work evidences a new mechanism that likely plays a critical role during the primary infection, the persistence but also the reactivation of BKPyV.

IMPORTANCE

Reactivation of BKPyV is responsible for nephropathies in kidney transplant recipients, which frequently leads to graft loss. The mechanisms of persistence and immune evasion used by this virus remain poorly understood and therapeutic option for transplant patients is still lacking. Here we show that BKPyV can be released into EVs enabling viral particles to infect cells using an alternative entry pathway. This provides a new view of BKPyV pathogenesis. Even though we did not evidence any
decreased sensitivity to neutralizing antibodies when comparing EV-associated particles and naked virions, our study also raises important questions about developing prevention strategies based on the induction or administration of neutralizing antibodies. Deciphering this new release pathway could enable the identification of therapeutic targets to prevent BKPyV nephropathies. It could also lead to a better understanding of the pathophysiology of other polyomaviruses that are associated with human diseases.

KEYWORDS

BKPyV, polyomavirus, extracellular vesicles, neutralizing antibodies, quasi-enveloped virus, en bloc transmission, JCPyV, MCPyV, TSPyV, SV40
INTRODUCTION

Most people are asymptomatic carriers of the BK polyomavirus (BKPyV). After contraction in the early childhood, the virus establishes persistent infection in the kidney and urogenital tract epithelial cells but the mechanisms of persistence and immune evasion remain poorly understood. BKPyV can also be reactivated and induce various complications in some patients, especially in case of immunosuppression. Reactivation of BKPyV is thus responsible for hemorrhagic cystitis in up to 15% of bone marrow transplant recipients and for nephropathies (BKVN) in up to 10% of kidney transplant recipients, which frequently leads to graft loss (1). Currently, the only therapeutic option for kidney transplant patients is to modulate immunosuppressive treatment in order to control infection, but this increases the risk of transplant rejection. Recent studies have suggested that patients with high neutralizing antibodies titers against the replicating strain had a lower risk of developing BKPyV viremia and that pre-vaccination against all serotypes, might offer protection against graft loss or dysfunction due to BKVN (2, 3). However, such a vaccine is still lacking.

A better understanding of the BKPyV life cycle could permit to identify new therapeutic targets to inhibit virus replication (4). In particular, only few studies were dedicated to understand the mechanisms of virion assembly and release. After translation, the VP1, VP2 and VP3 capsid proteins are translocated into the nucleus to assemble with viral genomes and form progeny virions (5). Then, naked virions are expected to be released after cell lysis. However, lytic infection is questionable in vivo, since the virus establishes infection that persists for life in healthy immunocompetent carriers. Furthermore, Evans et al. recently provided evidence for a non-lytic release pathway of BKPyV virions (6).

Here, we show that the BKPyV can be released within extracellular vesicles (EVs). We call these virus-containing vesicles “enveloped BKPyV” (eBKPyV). We also demonstrate that these
eBKPyV do not interact with cell surface sialylated glycans and compare their sensitivity to neutralizing antibodies with that of naked particles. This mechanism likely plays a major role in viral persistence.

RESULTS

BKPyV particles are released within EVs. Evans et al. recently showed that endosomes were involved in a non-lytic BKPyV release pathway (6). On our side, by performing electron microscopy on chronically infected Vero cells, we observed the presence of viral particles in the multivesicular bodies (MVBs), a specialized subset of endosomes (Fig. 1A). Since MVBs can fuse with the plasma membrane to release exosomes, we hypothesized that the non-lytic BKPyV release pathway could involve EVs. We thus decided to characterize the infectious particles released by infected Vero cells. Using iodixanol gradients, we observed the existence of two populations of BKPyV infectious particles (Fig. 1B). The population with the higher density peaked at 1.18 g/mL and likely corresponded to naked virions, coherent with the densities reported for the related JCPyV and MCPyV in such gradients (7, 8). In contrast, the second population of infectious particles, called eBKPyV, exhibited a density ranging between 1.05 and 1.15 g/mL, which was consistent with membrane association. We confirmed that this population co-sedimented with EVs by assessing the acetylcholinesterase (AChE) activity (Fig. 1B) as well as the presence of the tetraspanin membrane proteins CD9, CD63 and CD81, known to be enriched in these vesicles (Fig. 1C). We also considered the co-sedimentation with contaminating small cellular organelles by assessing the presence of GM130 and Calnexin. However, we observed that these contaminants peaked in fractions 5 and 6 contrary to EVs and infectious eBKPyV which peaked in fractions 7 and 8 (Fig. 1C). Importantly we easily detected the presence of the VP1 capsid
protein not only in the naked BKPyV fractions but also in the eBKPyV fractions, strongly suggesting
the presence of full viral particles in both types of fractions (Fig. 1C). We also demonstrated that this
phenomenon was not cell-type specific since similar results were obtained when working with primary
human renal proximal tubule epithelial (HRPTE) cells, which are a more physiologically relevant
model to study BKPyV infection (Fig. 1D).

As shown in Fig. 1E, when performing a proteinase K protection assay, which is commonly
used to study the envelopment of viruses, we observed that the eBKPyV VP1 was less sensitive to
proteinase K digestion than the naked BKPyV VP1 (Fig. 1E), suggesting that infectious particles of the
eBKPyV fraction were within vesicles. We also investigated the effect of chloroform extraction which
is a classical method to distinguish enveloped from non-enveloped viruses. As shown in Fig. 1F, the
treatment of eBKPyV with chloroform only had a slight effect on their infectivity, excluding the
possibility that naked BKPyV genomic DNA was transmitted to naive cells through EVs and
confirming that infectious virions were present in this fraction. Furthermore, when the chloroform
treatment was performed on the infected cell supernatants prior to the iodixanol gradient, we observed
the disappearance of the eBKPyV population and a slight increase of the naked BKPyV population
(Fig. 1G), consistent with enveloped particles rendered naked by chloroform treatment prior to the
gradient. Thus, our results strongly suggested that infectious viral particles were contained within EVs.

To firmly confirm that eBKPyV were contained into EVs and not stuck to them, we performed
electron microscopy. To get a sufficient amount of viral particles in the different fractions, we carried
out a polyethylene glycol (PEG) precipitation prior the iodixanol gradient. Vero cells were then
incubated with eBKPyV- or naked BKPyV-enriched fractions, fixed and processed for electron
microscopy. As expected, isolated or grouped naked particles were clearly observed at the surface of
Vero cells incubated with naked BKPyV enriched fractions (Fig. 2A and 2B). In contrast, vesicles
carrying one or more tens of viral particles were almost exclusively observed when cells were
incubated with eBKPyV enriched fractions (Fig. 2C and 2D). Similar vesicles but free of virions were observed with the corresponding fractions obtained from the supernatant of non-infected cells (Fig. 2E and 2F).

eBKPyV do not interact with gangliosides at the cell surface. Several studies have suggested that polysialylated receptors, in particular gangliosides, play an important role in the initial interaction between BKPyV and target cells (9, 10). Since eBKPyV are surrounded by a lipid bilayer, we hypothesized that they use an alternative entry pathway to infect target cells, as compared to naked BKPyV. This hypothesis was supported by electron microscopy experiments which clearly demonstrated not only the docking of membrane-wrapped viral particles at the plasma membrane of target cells (Fig. 2C and 2D) but also the presence of intact vesicles carrying virions in endosomal compartments (Fig. 3A). It has been shown that naked BKPyV agglutinate human type O red blood cells (RBCs) through interactions between the VP1 capsid protein and the gangliosides displayed at the surface of these cells (11). We thus investigated whether eBKPyV were able to agglutinate human type O RBCs. As expected, we observed that naked BKPyV agglutinate RBCs and that chloroform treatment had no effect on this ability (Fig. 3B). In contrast, viral particles contained in the eBKPyV fraction were able to agglutinate RBCs only after extraction with chloroform suggesting that they do not use polysialylated gangliosides as attachment factor. To confirm this result, we treated naive Vero cells with increasing concentrations of neuraminidase prior to incubation with eBKPyV or naked BKPyV. As shown in Fig. 3C, neuraminidase treatment efficiently inhibited naked virion entry in a dose-dependent manner whereas it only had a slight effect on infection with eBKPyV. These findings indicate that the entry of eBKPyV is not dependent on the presence of cell surface sialic acids.
**DISCUSSION**

In this study, we demonstrated that BKPyV uses EVs to be released from infected cells. In recent years, a similar strategy has been described for several viruses that had long been considered as non-enveloped, such as hepatitis A and E viruses, Coxsackievirus, Poliovirus, Rotavirus or Norovirus (12-19). The possibility for naked viruses to be released in EVs confers them several advantages: i) the possibility to be released from infected cells through a non-lytic pathway, ii) a diversification of the
transmission routes which promotes the propagation, iii) an enhancement of the virulence and viral fitness thanks to \textit{en bloc} delivery, and iv) a protection against neutralizing antibodies which target the viral capsid (17-21). Interestingly, it has been shown in 1989 that the release of SV40 virions from epithelial cells was polarized and occurred without cell lysis (22) and we also observed the release of two populations of SV40 infectious particles, one of which co-sedimenting with EVs (data not shown).

In addition, during the preparation of our manuscript, Morris-Love \textit{et al.} provided evidence that the JC polyomavirus (JCPyV), which shares 75\% sequence homology with BKPyV, also uses EVs as a means of transmission (7). Thus, several members of the polyomavirus family hijacks EVs for their release and the question is raised for other polyomaviruses such as MCPyV or TSPyV, which are associated with Merkel cell carcinoma and Trichodysplasia spinulosa, respectively.

The mechanism leading to the release of eBKPyV has not been deciphered yet. Using the anion channel inhibitor DIDS, Evans \textit{et al.} described that BKPyV is released by a non-lytic pathway and we think that this may correspond to the release of eBKPyV. Importantly, this mechanism could contribute to the asymptomatic persistence of BKPyV in immunocompetent individuals. We noticed that eBKPyV and naked BKPyV were released by HRPE cells from 5 days post-infection whereas cell lysis was observed from 15 days post-infection. Furthermore, when testing the effect of DIDS in our model, we observed that it inhibited not only the release of eBKPyV but also that of naked BKPyV (data not shown). Altogether, these results suggest that most naked BKPyV could come from the disruption of the membranes surrounding eBKPyV. However, we cannot exclude that the lysis of just a few infected cells could be responsible for the release of naked virions and in our hands, the toxic concentration of DIDS was too close to the effective concentration to draw firm conclusions. Further studies are thus needed to fully elucidate the mechanism leading to the release of eBKPyV and naked BKPyV. Previous studies have shown that some viruses hijacking EVs use the ESCRT machinery to bud into MVBs (14, 20, 21, 23-25). Since we observed the presence of viral particles in MVBs, it is tempting to speculate
about the involvement of this machinery in the release of eBKPyV. Alternatively, the autophagy process could be involved as described for other viruses (15, 16). The investigation of eBKPyV release pathway could enable the identification of new therapeutic targets to prevent BKVN. For instance, it has been proposed that targeting exosome biogenesis and release may have potential clinical implications for cancer therapy (26). The interaction between viral proteins and the ESCRT machinery has also been proposed as a potential target for antiviral therapy to fight against enveloped viruses (27, 28) but also EV-associated naked viruses (29).

Naked BKPyV are known to use gangliosides for their attachment and entry into target cells (10, 30). In contrast, we clearly evidenced by electron microscopy that eBKPyV were able to dock at the plasma membrane and to be endocytosed without interacting with cell surface sialylated glycans. Instead, the lipids of EV membranes (e.g. phosphatidylserine) may play a role but this remains to be demonstrated (19). Thus, naked virions and eBKPyV use different entry pathways, which could play a critical role in the dissemination and spread of BKPyV not only during the primary infection but also during BKVN. Such an alternative mechanism of infection has also been elegantly demonstrated for EV-associated JCPyV (7). Besides, it has been proposed that this plays a critical role in the dissemination and spread of JCPyV both to and within the central nervous system (7, 31).

For some viruses en bloc delivery enables to enhance the specific infectivity and viral fitness of viruses thanks to genetic cooperativity among viral quasispecies (17-19). We did not observe such an increased specific infectivity (i.e. infectivity normalized to VP1 content) in our model. However, it is important to note that we used the Dunlop strain which contains a rearranged non-coding control region and is highly adapted to cell culture. From our point of view, it would be more suitable to investigate a potential enhancement of the viral fitness in the context of an archetypal strain.

Some studies have evaluated the benefit of administering intravenous immunoglobulin preparations containing high titers of potent BKPyV neutralizing antibodies to patients, in conjunction
with reduced immunosuppression (32). However, these clinical studies are difficult to evaluate because of many caveats such as the existence of other concurrent antiviral interventions or widely variable, empirical dosing (32). It has also been suggested that pre-vaccinating prospective kidney transplant recipients with a multivalent virus like particles based vaccine against all serotypes might offer protection against graft loss or dysfunction due to BKVN (2). We expected that the membranes surrounding eBKPyV protect them from neutralization by antibodies. Such results were obtained with EV-associated JCPyV by Morris-Love et al. (7). In contrast, we did not evidence any difference when we compared the sensitivity of eBKPyV and naked BKPyV to neutralization, by performing dose-response curves with the serum of a seropositive patient or commercially available IVIg preparations. However, we observed that the naked and enveloped BKPyV were neutralized by the serum up to 4 h after inoculation, suggesting that a post-attachment step was blocked by neutralizing antibodies. Thus, it is likely that neutralization occurs after co-internalization and vesicle membrane disruption, as already shown for other viruses such as HAV (14, 19). We plan to decipher the mechanisms by which neutralizing antibodies inhibit eBKPyV and naked BKPyV but an in depth study will be required to identify the precise entry step that is targeted and to demonstrate that a membrane rupture step occurs for eBKPyV. In any case, our study provides a new view of BKPyV pathogenesis, which raises important questions about the prevention strategies that are based on the induction or administration of neutralizing antibodies. eBKPyV will have to be considered for future studies on BKPyV neutralization.
MATERIALS AND METHODS

Cell culture. Vero (CCL-81) cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS). HRPTE cells were obtained from Clinisciences (4100-sc) and cultured in Renal Epithelial Cell Growth Medium (REGM™, Lonza). All cells were grown at 37°C in a humidified environment with 5% CO2.

Antibodies and reagents. The anti-AgT mouse monoclonal antibody (PAb416) was purchased from Abcam. The 3B2 monoclonal anti-BKPyV VP1 antibody, the anti-mouse IgG (whole molecule)–peroxidase antibody produced in rabbit and the neuraminidase were purchased from Sigma. The Alexa Fluor Plus 488-conjugated goat anti-Mouse IgG (H+L) was purchased from Thermofisher. The monoclonal anti-CD63 antibody (MX-49.129.5), the monoclonal anti-Calnexin antibody (AF18) and DAPI were purchased from SantaCruz Biotechnology. The monoclonal anti-CD81 antibody (5A6) was kindly provided by J. Dubuisson (Center for Infection and Immunity of Lille, France). The polyclonal anti-CD9 antibody (GTX55564) was purchased from GeneTex. The monoclonal anti-GM130 antibody (35/GM130) was purchased from BD Biosciences.

BKPyV production. The plasmid BKV-pUC19 (kindly provided by WJ. Atwood, Brown University, USA) was used to produce the BKPyV. It was obtained from pBKv(34-2) (Dunlop strain, genotype I) as described previously (33). The plasmid was digested with 2U of BamHI (New England Biolabs) for every 1 µg of DNA for 4 h at 37°C to separate the BKPyV genome from the backbone plasmid. The DNA was then incubated at 65°C to inactivate the enzyme and it was transfected into Vero cells using Lipofectamine (Invitrogen), as described in the manufacturer’s instructions. Cells were cultured for
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approximately 4 weeks until a cytopathic effect was observed. Then, BKPyV was amplified by successive infections of naive cells at a multiplicity of infection (MOI) of 1, every four days. Extra and intracellular viral particles were harvested, extracted by chloroform treatment (34), and filtered at 0.45 µm. The titers of viral stocks were determined by the 50% tissue culture infective dose (TCID<sub>50</sub>) method using immunofluorescence (see below). To produce eBKPyV and naked BKPyV, Vero or HRPTe cells were infected with the BKPyV at a MOI of 1. The supernatants were harvested several days post-infection, filtered at 0.45 µm and overlaid on iodixanol gradients as described below.

Buoyant density iodixanol gradient ultracentrifugation. The supernatants of infected cells were overlaid on iodixanol gradients formed by equal volume (2.3 mL) steps of 20, 25, 30, 35, 40 and 45% (w/v) iodixanol (Visipaque 320 mg/ml, GE Healthcare) solutions in Phosphate-buffered saline (PBS). Equilibrium was reached by ultracentrifugation for 24 h at 130 000 g in an SW32.1 Ti rotor at 4°C in a Beckman OPTIMA L-100 K BioSafe ultracentrifuge. Seventeen fractions (1 mL) were collected from the top. The density (g/mL) of each fraction was calculated according to the optical density at 340 nm. AChE activity was measured using the Ellman’s method to detect the presence of EVs in each fraction (35). Briefly, 50 µL of each fraction were incubated at 25°C for 15 min with 150 µL of Ellman solution containing 1 mM acetylthiocholine iodide, 0.23 mM 5,5′-Dithiobis(2-nitrobenzoic acid) and 0.45 mM NaHCO<sub>3</sub>, all purchased from Sigma. The absorbance was measured at 405 nm. Infectivity of each fraction was assessed as described below.

Infectivity assays. Twenty microliters of the fractions recovered after iodixanol gradients were incubated with Vero cells in 96-well plates. At 72 h post-infection, cells were washed with PBS, fixed with paraformaldehyde (3.7 % in PBS) and permeabilized with Triton-X100 (0.05 % in CSK buffer). Infected cells were detected by immunofluorescence staining of the AgT. Nuclei were stained with
DAPI. Immunostained cells were observed with a Zeiss Axio Vert.A1 microscope equipped with Colibri 7 LED Illumination. Fluorescent signals were collected with a Axiocam 305 color camera (Zeiss). Percentages of infected cells were automatically determined using the QuantIF ImageJ Macro (36).

**Proteinase K protection assay.** Fractions of interest were treated with different concentrations of proteinase K (Qiagen) on ice during 10 min. The reaction was stopped by addition of a 10X solution of protease inhibitor (Pierce Protease Inhibitor Tablets) and by 2x Laemmli buffer (Sigma). The VP1 capsid protein was then detected by western blot using the 3B2 monoclonal anti-BKV VP1 antibody (Sigma). The bands were quantified using ImageJ.

**Electron microscopy.** Viral particles contained in the supernatants of infected cells were concentrated 100 X using PEG precipitation. Briefly, 300 mL of infected cell supernatant were mixed with 75 mL of a PEG-6000 solution (40% in PBS). The supernatant of naive cells was used as a negative control. The mixtures were incubated overnight at 4°C and centrifuged at 1500 g, 4°C for 30 min. The pellets were resuspended in 3 mL of PBS and fractionated by iodixanol gradient as described above. Cells were trypsinized and incubated with the fractions of interest during 2 h at 37°C, under gentle shaking. Then, they were washed with PBS, fixed in 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 48 h and postfixed with 2% osmium tetroxide (Electron Microscopy Science, Hatfield, PA, USA) for 1 h. They were then dehydrated in a graded series of ethanol solutions, and cell pellets were embedded in Epon resin, which was allowed to polymerize for 48 h at 60°C. Ultrathin sections were cut on an ultramicrotome (Reichert, Heidelberg, Germany), collected on copper grids, and stained with 5% uranyl acetate–5% lead citrate. The grids were observed with a JEOL JEM-1011
electron microscope (JEOL, Tokyo, Japan) connected to a GATAN RIO9 digital camera driven by Digital Micrograph software (Gatan, Pleasanton, CA, USA).

Hemagglutination assays. RBCs from type O Rh+ blood-donors were washed three times and suspended in PBS at a final concentration of 0.67% (v/v). Then, 50 µL of the suspension was mixed with 25 µL of serial dilutions of eBKPyV or naked BKPyV fractions, previously extracted with chloroform or left untreated. Mixtures were allowed to settle overnight at 4°C, in round-bottom 96-well plates.

Neutralization assays. Naked BKPyV or eBKPyV were preincubated for 2 h at 37°C with serial dilutions of the serum of a seropositive patient (subtype Ia). Then, the mixtures were put in contact with target cells. Infectivity was assessed 3 days post-infection by immunofluorescence, as described above. The serum of a seronegative 1-year-old individual was used as a negative control. Similar experiment was also performed using increasing concentrations of polyvalent immunoglobulins (HIZENTRA® 200 mg/ml, CSL Behring).

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FIGURE LEGENDS

Fig 1. BKPyV particles are released within EVs. (A) Chronically infected Vero cells were fixed and processed for electron microscopy. Electron micrographs show the presence of viral particles (indicated by arrowheads) in MVBs. The right panel (scale bar = 100 nm) correspond to an enlargement of the left panel (scale bar = 0.2 μm). (B) Vero cells were infected with BKPyV at a MOI of 1. Supernatant was harvested 3 days post-infection, filtered at 0.45 μm, and overlaid on a 20 to 45% (w/v) iodixanol gradient. After a 24 h ultracentrifugation, seventeen fractions were collected. The density (g/mL) of each fraction was calculated according to the optical density at 340 nm. BKPyV infectivity in each fraction was assessed by immunofluorescence 3 days post-infection of naive Vero cells. It is expressed as percentages of infected cells. AChE activity was analyzed to detect the presence of EVs in each fraction. (C) EVs contained in the supernatant of infected cells was concentrated 100X by PEG precipitation and overlaid on a 20 to 45% (w/v) iodixanol gradient. The presence of EVs in fractions 1 to 13 was evaluated by the detection of CD9, CD63 and CD81 by western blot. The presence of viral capsids was evaluated by the detection of VP1. The presence of contaminating small cellular organelles was evaluated by the detection of GM130 and Calnexin. (D) The experiment was performed as in (B) with the supernatant of HRPTE cells, harvested 10 days post-infection. (E) Fractions containing eBKPyV (fraction 6) or naked BKPyV (fraction 12) were treated with different concentrations of proteinase K during 10 min. The volumes of the fractions were adjusted to treat similar amount of the VP1 protein in both conditions. The sensitivity to proteinase K digestion was then assessed by detection of the VP1 capsid protein by western blot (top panel) and evaluated by quantifying the relative amount of VP1 on the western blots using ImageJ (bottom panel). Results are reported as the means ± standard deviation of two independent experiments. (F) After iodixanol
gradient ultracentrifugation, fractions containing eBKPyV or naked BKPyV were treated with chloroform or left untreated and then analyzed for infectivity on naive Vero cells. (G) The supernatant of Vero cells was harvested 4 days post-infection and treated with chloroform or left untreated before performing the buoyant density iodixanol gradient ultracentrifugation. Results presented in panels B, D, F and G are means of duplicates from representative experiments.

Fig 2. eBKPyV correspond to EVs enclosing tens of viral particles. Vero cells were incubated for 2 h with fractions containing eBKPyV or naked BKPyV, fixed and processed for electron microscopy. (A, B) Electron micrographs of naked particles at the surface of Vero cells. (C, D) Electron micrographs of eBKPyV at the surface of Vero cells. (E, F) Electron micrographs of EVs free of virions at the surface of Vero cells. Naked particles and eBKPyV are indicated by arrows in panel A and C, respectively. The pictures B2, D2 and F2 correspond to enlargements of B1, D1 and F1, respectively.

Fig 3. eBKPyV do not interact with cell surface sialylated glycans. (A) Vero cells were incubated for 2 h with fractions containing eBKPyV, fixed and processed for electron microscopy. Electron micrographs show intact EVs containing viral particles in endosomal compartments (indicated by arrows). (B) After iodixanol gradient ultracentrifugation, fractions containing eBKPyV or naked BKPyV were treated with chloroform (Chl+) or left untreated (Chl-). Then, serial dilutions of treated and untreated fractions were mixed with human RBCs from a type O Rh+ blood donor and allowed to settle in round-bottom wells overnight at 4°C. PBS was used as a negative control for hemagglutination (T-). Results of a representative experiment are shown. (C) Naive Vero cells were treated with neuraminidase for 1 h at 37°C, at the indicated concentrations. Then, cells were washed and inoculated with fractions containing eBKPyV or naked BKPyV. Infection was assessed by immunofluorescence 2
days post-infection. Results are expressed as percentages of infection and are reported as the means ± 
standard deviation of six independent experiments.

Fig 4. eBKPyV are efficiently inhibited by neutralizing antibodies. (A) Fractions containing 
eBKPyV or naked BKPyV were preincubated for 2 h at 37°C with serial dilutions of a BKPyV 
seropositive patient serum. Then, mixes were put into contact with naive cells. Infectivity was assessed 
72 h post-infection. Results are expressed as percentages of neutralization and are reported as the 
means ± standard deviation of ten independent experiments. (B) Similar experiment was performed 
using the serum of a 1-year-old individual seronegative for BKPyV. (C) Similar experiment was 
performed using increasing concentrations of IVIg. Results are expressed as percentages of 
neutralization and are reported as the means ± standard deviation of two independent experiments. (D) 
Naked BKPyV or eBKPyV were inoculated to naive cells and IVIg was added at different time post-
inoculation (final concentration 2 mg/mL). Infectivity was assessed 48 h post-infection. Results are 
expressed as percentages of neutralization and are reported as the means ± standard deviation of six 
independent experiments.
FIG 2

naked BKPyV

C

eBKPyV

E

negative control

B1

D1

F1

B2

D2

F2

FIG 2
FIG 3
FIG 4
