Significance, cytomorphology of decoy cells in polyomavirus-associated nephropathy: Review of clinical, histopathological, and virological correlates with commentary

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Human polyomaviruses (PyV) are ubiquitous, remaining predominantly inactive hence asymptomatic in the healthy, immunocompetent population. BK and JC PyV potentially infect pan-urinary tract epithelial cells. With reactivation, PyV disrupt cell cycling mechanisms, facilitating viral replication leading to cell necrosis, exfoliation, and, infrequently, carcinogenesis. Exfoliated PyV-infected cells pose diagnostic pitfalls, hence they are termed "decoy cells" as they may mimic high-grade urothelial carcinoma cells. BK polyomavirus-associated-nephropathy (BKVAN) is an inflammatory disease causing interstitial fibrosis with tubular atrophy in renal transplant recipients, increasing risk of graft loss. BKVAN is confirmed by renal biopsy, and managed by immunosuppression modulation. As voided urine may provide pano-reno-urinary tract sampling, cytopathology may serve a critical diagnostic purpose coupled with decoy cell quantification and indirect BK PyV load gauging. Thus, identification of decoy cells and differentiation from high-grade urothelial carcinoma cells, and degenerated, benign urothelial cells, is clinically essential. PyV virology and pathobiology in the context of renal transplantation, immuno-suppression and BKVAN, and, decoy cell cytomorphology and cytopreparation with commentary are highlighted. Decoy cell overall characteristics: variable degeneration; cytomegaly; comet-like shapes; angular cytoplasmic extensions; eccentric, polar nuclear placements; moderate anisocytosis; typically single cells with high N:C ratios.

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Human polyomaviruses

Background

Studies by Shope and Hurst in 1933\(^1\) showed skin papillomas may be transmitted between wild cottontail rabbits using homogenized cell-free extracts from wart tissue, suggesting a contagious nature and likely viral etiology of skin warts. Subsequent studies reported wild cottontail rabbit papilloma viruses may induce metastatic skin cancers in domestic rabbits after pretreatment with tar in laboratory experiments. From these findings, the Shope papillomavirus\(^1\) was purported as being a carcinogenic agent requiring another promoting factor to ultimately induce malignant skin transformation in non-target tissues. Advanced molecular DNA hybridization and polymerase chain reaction (PCR) techniques developed in the 1970s subsequently revealed the plurality, species-specificity, and pathobiology of the papillomaviruses.\(^2\)

Work by Gross in 1953\(^3,4\) to isolate tumor-inducing DNA viruses revealed the Murine polyomavirus (MPyV) in mice. The term polyoma implies “many tumors”. The prototypical primate Simian vacuolating virus (SV40) was seen in monkey kidney cells by Sweet et al in 1960.\(^3,5-7\) MPyV infections induced salivary gland tumors in mice, whereas SV40 caused tumors in hamsters and transformed cultured human cells manifesting marked cytoplasmic vacuolization. These latter findings launched extensive research exploring the relationship between these DNA viruses and human cell transformation. The MPyV and SV40 viruses share similar transmission electron microscopy (TEM) characteristics and genomic features. They also typically remain inactive, benign, and symbiotic in their respective host cells, although these viruses manifested...
tumorigenic potential in experimental animals when immunosuppressed or inoculated with large volumes of viral particles.\textsuperscript{3,7}

The first two human polyomaviruses (PyV) were both isolated independently in 1971. Gardner et al reported the BK polyomavirus (BKPyV) involved in renal allograft BK polyomavirus-associated nephropathy (BKVAN),\textsuperscript{8} and Padgett et al reported the JC polyomavirus (JCPyV) involved in progressive multifocal leukoencephalopathy.\textsuperscript{9} BK and JC viruses were so named after the initials of their reference patients.\textsuperscript{6-9}

The SV40 viruses have no established human disease association, although it is believed they entered the human pool through contaminated polio vaccines but rarely cause nephropathy, central nervous system disease, or neoplasms.\textsuperscript{2,6,7} SV40 viruses demonstrate remarkable homology with BKPyV and JCPyV. This homology has been exploited to develop immunohistochemical stains labeling the common large T-tumor antigens (LTag), now practically exploited to develop immunohistochemical stains labeling the papillomavirus genome appears on one of its two DNA strands. PyV genomes appear on both DNA strands.\textsuperscript{2}

The protein composition of the PyV particles consists of virus-encoded structural polypeptides synthesized during the late lytic viral cycle and histone proteins derived from the infected host cell.\textsuperscript{2,3} The core histone proteins that interact with the PyV genome produce a chromatin matrix structure similar to that of the host cell.\textsuperscript{2,3,6,7} The major virion protein 1 (VP1) accounts for nearly 75\% of the viral protein mass. The PyV virion capsid is composed of 360 copies of VP1 assembled in icosahedral structure with other proteins within the virion that interact with the VP1 outer shell proteins and the viral genomic chromatin.

Multilateral cross infections between non-target hosts do not typically occur as papovaviruses are characteristically genus-, species- and host cell-type-specific in nature. Their stringent tropism infers that equally specific modes of host infection and pathogenesis exist between these viruses and their respective target tissues. How PyV gain entry into the human body is not fully ascertained—neither are the biological mechanisms involved in cellular infection. Specific surface receptor sites are thought to facilitate entry of viruses into the cells via endocytosis.\textsuperscript{2,3,6} PyV are likely absorbed via the upper respiratory tract, whereas human papillomaviruses are acquired through squamous epithelial micro-trauma. The DNA of PyV has been isolated from tonsillar tissues in children, suggesting this to be the initial entry route; viruses may also be transmitted via previously infected donor organs (Table 1).\textsuperscript{2,3,6,10,28}

PyV maintain a life cycle based on induced lysis of the infected target cells to release internalized, copied viral particles into the immediate biological milieu.

PyV infections are typically latent and asymptomatic unless the host becomes immunocompromised, manifesting a biological advantage permitting viral reactivation.\textsuperscript{2,3,6} Studies suggest these viruses are internalized through an interaction between VP1 and specific cellular receptors. BKPyV utilize gangliosides for internalization.\textsuperscript{2,3,6} Viruses then traverse the endoplasmic reticulum with the nucleus being the endpoint, wherein viruses uncode allowing for genomic transcription of early messages. Viruses first produce the LTag, which prompts replication of the viral DNA molecule. With LTag transcriptional activation, VP1 is expressed and when incorporated with virion proteins 2

\begin{table}[h]
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\hline
\textbf{Table 1} & \textbf{BK Polyomavirus mode of transmission.} \\
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\textbf{Mode of transmission} & \textbf{BK Polyomavirus mode of transmission} \\
\hline
Urine & \\
\hline
Nasopharyngeal secretions & \\
\hline
Fecal, oral contamination (transmission of viral DNA through urban sewage) & \\
\hline
Blood, skin & \\
\hline
Trans-placental (mother to fetus) & \\
\hline
Donor kidney & \\
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(VP2) and 3 (VP3) facilitate encapsidation of copied viral genome with eventual viral replication.\textsuperscript{2,3,6}

**PyV Pathogenesis**

When reactivated within mitotically quiescent host cells, PyV invoke unscheduled cell cycling and launch S-phase mechanisms to provide the needed biological systems by which to facilitate viral genome replication. Host cell cycling initiation is achieved through expression of mitogenic proteins by viruses early in their lytic cycle. These mitogenic proteins are eventually destroyed along with lysis of the infected cell. With host cell disintegration, viral particles are shed. Therefore, PyV replication and transmission occur at the expense of the host cell through necrosis.\textsuperscript{2,3,6}

Alternatively, if expression of the early viral proteins becomes uncoupled from the cell-lysis mechanisms, host cell transformation may result due to failed, averted lysis. Ongoing expression of mitogenic proteins evidently predisposes continuous S-phase cycling, thus uncontrolled cell proliferation with potential tumorigenesis. PyV oncogenicity correlates positively with the likelihood and frequency of viral protein uncoupling. The simple biological nature of these small PyV may require ongoing molecular interactions with the infected host cell, and this may foster frequent errors in the cell lysis mechanisms. PyV have stringent coding potentials, however, therefore must interact favorably with the host cell regulatory processes to secure their life cycle and achieve viral copying and shedding.\textsuperscript{2,3,6,7}

BKPyV and JCPyV may establish persistent infections in the reno-urinary tract epithelia following primary exposure. These viruses consequently shed viral particles that are carried in the urine flow in low volumes as background viruria following viral reactivation and host cell disintegration. In healthy individuals, intermittent, episodic viral reactivation may lead to sporadic, self-limiting pathology detectable through viruria. Reactivation of BKPyV and JCPyV in permissive cells, however, such as those in immunocompromised patients, is characterized by significantly increased degrees of viruria; and only for BKPyV does degree of viruria correlate with degree of immunosuppression.\textsuperscript{2,3,6,10}

**BK polyomavirus-associated nephropathy (BKVAN)**

**Background**

The relationship between BKPyV reactivation and BKVAN in kidney transplantation with associated immunosuppression was reported in the 1980s.\textsuperscript{6,8} The mechanisms of BKPyV reactivation in the renal allograft are multifactorial and not fully known, though robust levels of active viral replication may ultimately lead to allograft injury with consequent graft failure. The clinical impact of BKPyV infection seems to depend on the type of allograft: (a) hematopoietic stem cell transplantation is associated with hemorrhagic cystitis, hematuria, and renal impairment; while (b) renal transplantation is associated with BKVAN in 2% to 10% of patients.\textsuperscript{8,11,28}

BKVAN is a virally induced tubulointerstitial inflammatory disease and a critical consideration in renal transplant patients for suspected renal graft dysfunction and ensuing loss.\textsuperscript{8,10,11,28}

**BKVAN histopathology**

Histologically, PyV cytopathic changes are noted within the nuclei of epithelial cells lining the renal tubules, Bowman’s capsule, and collecting ducts in the renal pelvis.\textsuperscript{8} The characteristic cytopathic features include cytomegaly, karyomegaly with varying degrees of glassy nuclear morphology, and basophilic intra-nuclear inclusions forming a smudged pattern (Fig. 1). Not infrequently, epithelial cells along the involved nephron may appear focally necrotic or exfoliating, resulting in naked basement membranes surrounded by a mononuclear interstitial inflammation composed of lymphocytes, plasma cells, and macrophages. Neutrophils may also be noted in severe cases. With chronic disease, the involved tubules may show marked atrophy with associated interstitial fibrosis.\textsuperscript{8,10}

![Figure 1](image) Histological examination of this kidney biopsy shows the characteristic intranuclear viral inclusions along with significant tubular atrophy and interstitial fibrosis (Hematoxylin and Eosin stain, 200×).
Immunohistochemical stains for SV40 LTag are useful to reveal PyV-infected epithelial cells (Fig. 2).

Three histologic stages have been defined to report BKVAN.29–32 Stage A is the earliest disease phase, often limited to the medulla. It is characterized by limited BKPyV cytopathic changes and few intranuclear inclusions. The interstitium shows minimal focal inflammation. In stage B, there is florid tubular epithelial cell injury with prominent intranuclear viral inclusions and conspicuous denudation of tubular basement membranes. The interstitium shows marked inflammatory cell infiltrations. Stage C is the late sclerosing phase. It is characterized by tubular atrophy and interstitial fibrosis, involving more than 50% of the sampled tissue biopsy. A histologic diagnosis of BKVAN ought to include the stage of disease, as this may have prognostic significance.30–34

**BKVAN epidemiology**

Recent studies report a seroprevalence for antibodies against BKPyV and JCPyV in the general, healthy, immunocompetent adult population to be 82% and 39%, respectively.6–8 These data widely suggest that PyV are ubiquitous and the majority of adults become exposed to BKPyV early in life, likely in childhood. The actual incidence values of seropositivity in adults may be higher, however, as this parameter wanes with advancing age.6–8,10,11,28

If BKVAN develops in renal transplant patients as a result of immunosuppression and BKPyV reactivation, the ensuing pathology may lead to graft failure and loss due to extensive interstitial fibrosis and tubular atrophy if the disease is undetected or untreated. The literature describes an associated 40% risk of graft loss in patients with BKVAN.6–8 Therefore, diagnostic confirmation of early BKVAN is critical. It is noteworthy that recipients of other xenotransplants do not develop similar BKPyV reactivations; additionally, BKVAN is rarely seen in native kidneys.6–8

The recent increase in BKVAN cases globally is believed to be attributed to strong, multidrug, new-generation immunosuppressive agents that are currently administered6–8 although no one specific agent has been documented or implicated for BKVAN development. The potency of immunosuppression ultimately correlates with its effects on the human T-cell mediated immune response predisposing PyV reactivation. Studies report an increased risk of BKVAN development in patients who switched from older, milder immunosuppressive agents to newer, more potent agents.6–8

**BKVAN treatment**

The cornerstone of therapy for BKVAN and BKPyV viremia is the reduction of total immunosuppression. For patients who are on a maintenance triple-agent immunosuppression regimen (i.e., calcineurin inhibitor [tacrolimus or cyclosporin]; an anti-metabolite [mycophenolate mofetil or mycophenolic acid]; and prednisolone), immunosuppression is reduced by complete discontinuation of the anti-metabolite and by decreasing the dose of the calcineurin inhibitor. The risk of interstitial fibrosis thus decreases with improved prognosis for graft function as measured through successive serum creatinine level assays.6–8,10,11,28

PyV reactivation precedes the clinical symptomatology of BKVAN. This phenomenon underscores the need for regular and structured post-transplant screening for early detection of the signs and symptoms of possible renal dysfunction.5–8 Nevertheless, standard markers for renal function are not sensitive for BKVAN and patients with low levels of BKPyV viremia are typically asymptomatic.

Studies of BKPyV viremia and viruria using real-time PCR revealed that (a) BKPyV replication commences within hours of transplantation; (b) viral replication peaks in the third month post-transplant; and (c) viral replication decreases in incidence in the sixth month. As such, current diagnostic algorithms stipulate frequent screening in the first year post-transplantation with a reduction in screening after 2 years post-engraftment.6–8 BKPyV reactivation with viruria in immunosuppressed patients may range between 0% and 62%.6–8,10,11,28

Inappropriately balanced immunosuppression may increase the risk of allograft rejection. Therefore, reliable laboratory investigations that may gauge viral load and its effects are essential. Although renal biopsy remains the gold standard for distinguishing between BKVAN and graft rejection, alternative surveillance of BKPyV viruria and viremia may facilitate early detection of PyV reactivation, replication, and virion shedding, hence foster early patient management.

**Figure 2** Immunohistochemical stain for polyomavirus large T-antigen (SV40) showing many positive tubular epithelial cell nuclei indicating virus-infected cells (Immunohistochemical stain for SV40 large T-Antigen, 100×).
Laboratory investigations

Renal biopsy

Renal allograft biopsy is the index investigation for the detection of BKPyV cytopathic changes in epithelial cells and the grading of pathology for the exclusion of likely graft failure due to BKVAN. Thus, histologic analysis of renal allograft biopsy is the gold standard to confirm BKVAN, against which the sensitivity of other diagnostic laboratory investigations are measured. In the early phase of the disease, however, the pathological changes may be focal, limited only to the medulla. Therefore, both BKVAN and its staging may be inaccurately reported if biopsy tissue sampling is sub-optimal. As such, in our setting we recommend at least 2 core biopsies that include tissue from the medulla to better exclude graft dysfunction. Furthermore, renal biopsy may be associated with false-negativity if normal parenchyma is biopsied or if BKVAN is focal.

Urinary cytopathology

Microscopic analysis of voided urine cytopreparations for PyV-infected cells is a non-invasive investigation by which to detect epithelial cytopathic changes. Given potential pan-urinary tract representation by voided urine samples, cytopathology may effectively gauge viral load indirectly. The major advantages include relative technical simplicity, rapidity, and minimal associated expenditure. Also, voided urine may be collected repeatedly over a course of time, thus maximize the likelihood for test positivity hence efficacy.

In our experience, optimal cytopreparation is of paramount importance to facilitate adequate qualitative and quantitative assessments. Urine samples ought to be received fresh and processed promptly with adequate fixation and Papanicolaou staining. Voided urine specimens may be processed using various cytopreparatory modalities engineered to salvage small cells from likely hypocellular samples.

Transmission electron microscopy (TEM)

Viral particles may be identified in PyV-infected epithelial cells using TEM, both inside nuclei and cytoplasm. TEM is a sophisticated and costly technology, thus not routinely utilized for PyV surveillance. Note that TEM may not distinguish between polyomavirus subtypes because of physical virion similarities, therefore TEM may not reveal BKPyV involvement specifically. Nonetheless, TEM is a useful platform through which to study viral particle architecture and organization, and extent of intracellular cytopathic damage in confirmed BKVAN cases. The use of TEM to study PyV involvement remains mainly a research and ancillary tool that may reveal and explain the nuclear morphology noted in PyV infected cells in histopathology and cytopathology.

Immunohistochemistry

Specific immunohistochemical stains have been developed to reveal BKPyV involvement in renal biopsies through staining for SV40 LTag, an epitope common between PyV subtypes (Fig. 2). Immunohistochemical technology has become routine and simple to perform using modern automated platforms, both on core tissue biopsies and cytologic material. Nonetheless, immunohistochemical assays may fail to reveal BKPyV involvement if tissue biopsy is sub-optimal due to focal renal disease.

Real-time quantitative PCR

Non-invasive and sensitive surveillance of BKPyV DNA or RNA may be achieved by molecular PCR technology to test for viruria or viremia. The sensitivity of PCR assays for BK viremia approaches 100% and the specificity varies between 88% and 96% (as measured against renal biopsy-confirmed BKVAN). As such, renal biopsy may be avoided if BKVAN may be confirmed through PCR. Background viruria reflecting latent or episodic, asymptomatic viral replication in the urothelium of healthy individuals may also be detected through PCR. In contrast, however, viremia is believed to develop only after robust BKPyV reactivation and replication whereby shed viral particles gain entry into the peritubular capillaries adjacent to infected and necrotic tubular epithelial cells secondary to epithelial injury and associated inflammatory cell infiltration. PCR is relatively expensive and labor-intensive, and thus may not be readily accessible.

Mass spectrometry

Mass spectrometry may detect various PyV-derived proteins directly from urinary samples, aiding in the surveillance of proteins expressed during the clinical phases of BKVAN. Mass spectrometry facilitates the study of VP1 protein forms to potentially reveal different PyV subtypes and the degree of the associated viral replication. This platform may further aid in the differentiation between BKVAN and acute renal rejection. Similar to real-time quantitative PCR, mass spectrometry is not a routine test for PyV detection.

Decoy cell cytomorphology

Background

In the Atlas of Exfoliative Cytology (1954), George N. Papanicolaou described unusual urothelial cells (Fig. 3) as having:

"...certain characteristics suggestive of malignancy, such as unusually high nuclear-cytoplasmic ratio; They have been found in cases of malignancy as well as in cases in which no evidence of malignancy could be obtained;
One of their distinctive traits is the scantiness of the cytoplasm and its frequent concentration toward one pole of the cell; Their nature, origin and significance are therefore still obscure. In the late 1950s, Koss and coworkers launched a study of voided urine samples with respective tissue biopsies to determine the tumorigenic effects of para-aminodiphenyl exposure in workers followed for bladder cancer development. In some cases from that study, Andrew Ricci, a collaborating diagnostic cytologist, noted cells with enlarged and homogeneous nuclei resembling malignant urothelial cells, but generally lacking coarse nuclear chromatin. As Ricci regarded these unique cells to be of benign etiology, he introduced the term “decoy cell” to stress a potentially major diagnostic distraction manifesting a significant interpretive pitfall. Koss described the cytomorphology of these odd urothelial cells in 1968, emphasizing the clinical
risks in their misinterpretation, although neither their pathobiology nor clinical significance were known at that time. The term decoy cell then inferred a cytologic oddity and diagnostic pitfall.

The association between decoy cells and BKPyV infection was established by Gardner et al in 1971. Coleman and coworkers later described the cytomorphology of decoy cells in 1975, and revealed the intracellular viral particle characteristics in inclusion-bearing cells through TEM. TEM micrographs from Coleman’s studies of PyV inclusions correlated well with nuclear morphology as seen in PyV-infected cells in both renal histopathology and urinary cytopathology.

The term decoy cell is now established in modern cytopathology practice to report polyomavirus-infected urinary tract epithelial cells (i.e., polyomavirocytes). In keeping with Ricci’s rationale, however, the term also raises caution for possible misinterpretation arising from shared cytomorphologic features between cells of unlike origin. Cytomorphologic overlap may occur between decoy cells and other clinically relevant cells that may resemble them, such as high-grade urothelial carcinoma cells, or benign, degenerated urothelial cells, depending on what cytomorphologic features may be shared between these simulating cell types.

BKPyV and JCPyV may infect epithelial cells from glomeruli through to bladder, and both viral subtypes have been identified in urine and brain tissue. Therefore, the decoy cell cytomorphologic template may be relevant for both BK and JC polyomavirocytes arising from any locus throughout the urinary tract. Unlike surgical biopsy, voided urine cytopathology may sample the entire urinary tract and because of this expanded degree of representation may prove sensitive in revealing PyV involvement through the detection of urinary decoy cells. Hence an awareness of the differential cytomorphologic features and the clinical significance of decoy cells in the context of renal transplantation, immunosuppression, and BKVAN is important.

We emphasize that the term decoy cell applies exclusively to epithelial cells exhibiting characteristic cytomorphology in urinary specimens. Regardless whether such cells are of renal tubular or bladder urothelial origin, the decoy cell cytomorphologic template includes specific nuclear and cytoplasmic features, and overall cellular presentation characteristics. These features may ultimately reflect the cytopathic effects arising from PyV pathobiology, the physiological effects of urine toxicity, and the artifactual effects of cytopreparation.

**Decoy cell nuclear features**

Decoy cell nuclear features discerned cytologically closely mirror those described through renal histopathology and TEM. The amount and organization of intranuclear PyV particles in decoy cells seem to predispose the cell’s nuclear morphology as seen through light microscopy and Papanicolaou staining. An important consideration may also be the histone proteins within the PyV virions derived from the host cell. TEM has shown that intranuclear viral particles may be haphazardly scattered and unorganized or precisely organized within crystalline lattices. Cytologically, when viral particles are scattered between small clumps of chromatin material, and depending on their amount and location, nuclei may appear to contain haphazard chromatin densities (akin to beads) (Figs. 4 and 5). In contrast, if viral particles are numerous and packaged into crystalline lattices, this evidently results in the characteristic homogeneous, dense, ground glass nuclear morphology (Figs. 4—6). Depending on the mass and organization of crystalline lattices, displaced chromatin densities may appear to be beading alongside the inner periphery of the nuclear envelopes (Figs. 4 and 5).

In our experience, decoy cell nuclei often appear glassy yet opaque because of their lack of transparency (i.e., amorphous with a metallic sheen). This we attribute to the tightly organized lattices of viral particles composed predominantly of VP1 that presumably interfere with the transmission of light despite optimal Kohler illumination settings. The crystalline lattices lack discernable staining patterns even with optimal fixation and staining (Fig. 6). Alternatively, the nuclear ground glass masses may appear diffusely dark blue-gray, reminiscent of a dark blue

![Figure 4](image-url)  Decoy cells. Moderate anisonucleosis, regular nuclear envelopes, symmetrical nuclear shapes, karyomegaly, high N:C ratios, chromatin beading, ground glass morphology, elongated comet-like cellular shapes, angular cytoplasmic extensions (Cyto-centrifugation, Papanicolaou stain, 400×).
ink drop filling up the nuclear space. For practical purposes, we describe this phenomenon as “inking of the nucleus” during our teaching sessions to emphasize this phenomenon.

Decoy cells have characteristically enlarged nuclei, approximately twice the size of a benign non-surface urothelial cell nucleus (Figs. 6 and 7). The degree of karyomegaly may reflect the overall mass of intranuclear viral particles; but may also be due to generalized cellular swelling resulting from cellular degeneration due to edematous osmotic stress in the disease process. Urine tonicity may also lead to karyomegaly. We do not regard decoy cell karyomegaly to be an artifact of cytopreparation unless cells were air-dried prior to Papanicolaou staining.

Decoy cell nuclear envelopes typically have symmetrical axes with minimal contour wrinkling or irregularities. With optimal Papanicolaou staining, nuclear chromatin densities are distinct but may stain markedly hyperchromatic, thus simulate coarse chromatin clumps as those seen in malignant cells (Figs. 8-10). Nevertheless, coarse chromatin clumps and marked nuclear irregularities are typically absent in decoy cells. In our experience, decoy cell nuclei may also appear pyknotic (Figs. 4 and 5), and occasionally include small, regular nucleoli. We emphasize the importance of adequate staining and microscopic inspection of nuclear detail using high magnification.

Decoy cell cytoplasmic features

Decoy cells demonstrate minimal to moderate anisocytosis with predominantly round to oval shapes. But they may appear enlarged, assuming elongated comet-like shapes, as their nuclei may have plasmacytoid (eccentric, polar) placements (Figs. 4—8). We surmise comet-like cytomorphology results from partial loss of cytoplasm. This presumption may be supported by the angular cytoplasmic extensions oftentimes observed in elongated decoy cells, and the cytoplasmic micro-debris in the backgrounds of positive cases (Figs. 7 and 8).

Decoy cell cytoplasm is typically moderately abundant and granular, revealing a diffuse, ill-defined reticular morphology without perinuclear clearing or halos (Figs. 4—6 and 8). The obscure cytoplasmic features may reflect the effects of viral particles in the cytoplasm due to ruptured nuclear envelopes, as has been revealed by TEM. With Papanicolaou staining, decoy cell cytoplasm yields variable monochromatic shades of blue-gray; arguably reflecting equally variable degrees of cellular degeneration. Cells with marked degenerative changes tend to stain lighter gray with bland overall cytoplasmic morphology. We correlate lighter cytoplasmic staining with decreased cellular viability. Depending on the case, decoy cells may occasionally appear as faint amorphous objects reflecting complete cytoplasmic lysis leading to naked nuclei (Figs. 4 and 5). Air-drying prior
to fixation and Papanicolaou staining may further obscure the cytoplasmic features in decoy cells. As Diff-Quik staining may hinder visualization of decoy cell features it is not recommended for routine diagnostic practice.

Decoy cells typically exhibit high nuclear to cytoplasmic ratios (N:C). This assessment depends on the amount of cytoplasm evident cytologically. Occasionally, disintegrated decoy cells may appear as random aggregates of naked nuclei among variable fragments of cytoplasmic debris (Figs. 7 and 8). The extent of decoy cell degeneration or necrosis may reflect a combination of the degree of cellular viability arising from the disease process, and the physiological effects of urine toxicity and tonicity upon suspended cells. Because of the presence of proteolytic enzymes and bacterial cytolysins in urine, suspended urothelial cells, particularly when isolated, may deteriorate rapidly with degenerative changes being apparent within one hour of voiding.57

In the context of BKVAN, cytologic assessment of decoy cell degeneration may prove clinically useful to aid in disease grading. Cytologists ought to take into account any lapsed time between specimen collection and cytopreparation to better assess the likely significance of the observed cytomorphology.

**Overall decoy cell presentation**

Under low microscopic magnification, decoy cells appear relatively monomorphic, exhibiting monochromatism and moderate anisocytosis and anisonucleosis (Figs. 4 and 5). They also appear predominantly singly in cytopreparations (Figs. 4–8). In our experience, intimate, cohesive clustering is rarely identified. These cells seem to exfoliate singly, hence present isolated, presumably due to degeneration and lack of cell junctions. When clustering is noted, it may be artifactual secondary to cytopreparation.

Although decoy cells may be seen against clear backgrounds, particularly if voided urine specimens are washed using cytopreparatory reagents to clear precipitates, they are predominantly seen against granular backgrounds and fragments of cytoplasmic debris. We argue that such backgrounds ultimately reflect the nature of PyV pathobiology, particularly in specimens from immunocompromised patients with advanced-stage BKVAN and associated florid cellular necrosis (Fig. 8). Erythrocyte membranes may be identified in the background of decoy cells, particularly in cases with hematuria and if cytopreparations are subjected to lyzing reagents.

During the microscopic screening process with low magnification, the contrasting overall characteristics of decoy cells include comet-like shapes, eccentric and polar nuclear placements, angular cytoplasmic extensions, relative monochromatism, and predominantly isolated single cells typically with high N:C ratios.
Diagnostic pitfalls

The basis of cytologic detection of decoy cells in urinary specimens stems from reactivation and replication of either endogenous PyV or those acquired from donor kidneys. Robust viral reactivation may result in focal or diffuse epithelial cell injury, necrosis, and ensuing exfoliation of infected epithelial cells in voided urine specimens. Based on their characteristic cytomorphologic features, decoy cells may be identified and reported with confidence. Any equivocal cytology ought to be reported conservatively, however, in conjunction with patient history and available clinical information. The need for fresh voided urinary samples with prompt delivery to the laboratory for cytoreparation cannot be overemphasized, nor the need for optimal fixation and Papanicolaou staining.

Cytomorphologic overlap may occur between decoy cells and urothelial cells reflecting other viral infections such as cytomegalovirus, adenovirus, and herpes simplex virus. Note, however, that neither the halos that oftentimes surround large intranuclear inclusions in the “owl-eye” appearance of cytomegalovirus-infected epithelial cells, nor the nuclear molding as seen in herpes simplex virus, are features seen in decoy cells.

Cytomorphologic overlap may pose diagnostic dilemmas, however, when considering decoy cells from malignant cells exfoliating from high-grade urothelial carcinoma. This problem is further confounded as decoy cells may also simulate benign, but degenerated, urothelial cells cytologically. The most useful cytomorphologic features that may discriminate between these simulating urothelial cells are the nuclear characteristics. Careful study of nuclear morphology is essential using high microscopic magnification with multi-plane focusing.

Benign, degenerated urothelial cells rarely show marked nuclear coarseness to raise suspicion of malignancy, although they may have bland, ill-defined, nuclear and cytoplasmic morphology depending on the extent of degeneration and staining reactions. Their nuclear chromatin patterns and distribution are typically vesicular and euchromatic. Careful study of nuclear morphology may resemble the ground glass patterns seen in decoy cells, although we have not noticed the metallic sheen characteristic of PyV in degenerated urothelial cell nuclei that also tend to have regular envelopes without evidence of chromatin beading. Moreover, benign urothelial cells, even if degenerated, generally have larger amounts of cytoplasm, thus lower N:C ratios. Both decoy cell and benign urothelial cell nuclei may rarely have nucleoli. But benign degenerated urothelial cell nuclei may contain chromocenters requiring careful microscopic scrutiny as they are generally small and likely to be mistaken for intranuclear inclusion bodies or chromatin beading.
More importantly, cytomorphologic overlap between decoy cells and high-grade urothelial carcinoma cells may pose significant diagnostic pitfalls leading to uncertain or erroneous interpretations. Urothelial carcinoma cells originating from high-grade lesions tend to appear singly, although small clusters can be noted. When single, these malignant cells are predominantly smaller than decoy cells and may demonstrate increased overall pleomorphism inclusive of marked anisocytosis with variable cytoplasmic staining (which may be dense). High-grade urothelial carcinoma cell nuclei demonstrate marked anisonucleosis and are characteristically hyperchromatic with coarse chromatin clumping (Figs. 9 and 10). Their nuclear envelopes are irregular, thickened, with marked wrinkling. High-grade urothelial carcinoma cells may have prominent and multiple nucleoli, and occasionally demonstrate squamous differentiation, inclusive of spindle cells.

Any clusters or pseudopapillary groups of atypical urothelial cells observed in optimal cytopreparations, even with coexisting decoy cells, raise the prospect of possible urothelial neoplasia and ought to be reported accordingly, recommending voided urine resampling and additional investigations.

Background detail may also aid in cell-type discrimination. Polyomavirocytes are usually seen against backgrounds containing granular precipitate. In contrast, high-grade urothelial carcinoma cells are typically noted against a background of necrotic cellular or proteinaceous debris, which may include fibrin deposition and erythrocytes in patients with invasive disease (Fig. 10). Therefore, erythrocytes per se, whether intact or lyzed, may not aid in the distinction between BKVAN and urothelial carcinoma, as both lesions may present with gross hematuria.

Table 2 summarizes decoy cell nuclear and cytoplasmic features using 95% ethanol fixation and Papanicolaou staining with overall presentation and background characteristics.

Urothelial PyV infections may undergo episodic reactivation with associated hemorrhagic cystitis. Lee et al reported that ICPyV infected decoy cells were more commonly associated with asymptomatic hemorrhagic cystitis in healthy individuals rather than with the BKPyV subtype. Also, they reported that such clinical episodes appeared with increasing frequency in men with advancing age. Both BKPyV and ICPyV may cause hemorrhagic cystitis and ureteral stenosis in immunosuppressed patients. Therefore, hemorrhagic cystitis is not unique to PyV infections and may pose clinical and diagnostic dilemmas in either immunosuppressed or healthy individuals.

Hemorrhagic cystitis due to bacterial infection needs to be considered and ruled out, yet another consideration is Boon’s disease. This entity is characterized cytologically by the identification of numerous apoptotic, degenerated urothelial cells of bladder origin secondary to epithelial denudation following phases of extreme dehydration. As apoptotic epithelial cells in Boon’s disease may demonstrate marked nuclear degeneration including chromatin condensation and smudging, they may pose additional cytodiagnostic challenges, particularly when considering the likely presence of decoy cells in the clinical context of renal transplantation, BKVAN, or immunosuppression. Moreover, similar cytomorphologic nuclear alterations have been reported in urothelial cells following cyclophosphamide treatment in oncology patients due to the effects of toxic metabolites upon bladder urothelium. These clinical considerations may compound the overall cytodiagnostic challenges pertaining to the identification of decoy cells or BKVAN, and particularly in immunosuppressed patients because of the likely pitfalls arising from expanded cytomorphologic overlap.

Utility of urinary cytopathology

Cytologic voided urine analysis is a suitable platform in the context of BKVAN, as it may indirectly gauge viral load in diseased patients. Unlike tissue biopsy, voided urine cytopathology may sample pan-reno-urinary tract PyV cytopathy. This test is well suited relative to other labor-intensive laboratory investigations such as PCR and TEM.

Cytopreparations ought to be qualitatively and quantitatively optimal. Current technology may produce adequate results despite degree of samplecellularity. Irrespective of mode of cytopreparation, however, adequate fixation and elimination of air-drying are universally important.

Figure 11  Benign, degenerated urothelial cells (Cytocentrifugation, Papanicolaou stain, 200×).
have PyV viremia. Note, however, that cytopa-
tients may exhibit decoy cell viruria, whereas 13% may
tool for gauging viral load as 10% to 30% of BKVAN pa-
logically can correlate with the degree of viral reactivation
quantitative analyses.

Cell-block sections may also be produced from hyper-
plasma through PCR. The number of decoy cells in urine
tionship between the number of decoy cells noted in urinary
tive surveillances and modulation of immuno-
suppression is critical and may be facilitated through
cytologic decoy cell quantification. This is particularly
important because only for BKPyV does degree of viruria
 correlate with degree of immunosuppression, and because
PyV reactivation precedes the clinical symptoms of
BKVAN.

Recent work by Huang and coworkers has demonstrated
the usefulness of monitoring patients post-transplantation
every 3 to 6 months. Using BKPyV DNA PCR assays and
urinary cytology samples, appropriate immuno-
suppression modulation resulted in favorable overall 5-
year patient and graft survival rates of 95.6% and 92.1%,
respectively. Huang et al also demonstrated a direct rela-
tionship between the number of decoy cells noted in urinary
cytology and the amount of BKPyV DNA identified in
plasma through PCR. The number of decoy cells in urine
related positively with BKVAN stages A, B, and C,
reflecting increasing severity and extent of cytopathic
disease. Detection of decoy cells in urinary cytology samples
without quantification had a sensitivity of 95.8% and
specificity of 83.1% for BKVAN. With quantification,
however, specificity for BKVAN increased to 99.7% using a
threshold of >20 decoy cells per 10 high power fields
(HPF). Huang et al further reported that the >20 decoy
cells per 10 HPF threshold correlated with BKVAN stage C
leading to a 92.9% positive predictive value for BKVAN.

With any future implementation of quantitative decoy
cell threshold analyses in routine practice, cytopreparatory
platforms may also need to be standardized to allow for
meaningful quantification and reporting of decoy cells. As
such, dilution factors may need to be dictated for hyper-
cellular samples, or concentration factors for hypocellular
samples, intended for cytocentrifugation. Therefore

<table>
<thead>
<tr>
<th>Nucleus</th>
<th>Cytoplasm</th>
<th>Overall presentation</th>
<th>Backgrounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate anisonucleosis, eccentric (polar) locations</td>
<td>Cytomegaly</td>
<td>Moderate anisocytosis</td>
<td>Granular debris</td>
</tr>
<tr>
<td>Haphazardly scattered chromatin densities (beads)</td>
<td>Round, oval, elongated, comet-like shapes</td>
<td>Predominantly single, isolated cells</td>
<td>Cellular debris</td>
</tr>
<tr>
<td>Chromatin densities displaced alongside inner periphery of thin, regular envelopes</td>
<td>Moderately abundant granular cytoplasm</td>
<td>Variable degrees of degeneration</td>
<td>Intact or fragmented renal tubular casts (typically granular type)</td>
</tr>
<tr>
<td>Dense, homogenous, basophilic ground glass masses</td>
<td>Angular cytoplasmic extensions</td>
<td>Relative cytomegaly (compared with benign non-surface urothelial cells)</td>
<td>Frequently intact or lyzed erythrocytes</td>
</tr>
<tr>
<td>Variable degrees of degeneration</td>
<td>Blue-gray staining</td>
<td>High N:C ratios</td>
<td>Infrequently inflammatory cells</td>
</tr>
<tr>
<td>Hyperchromasia</td>
<td>Lacking dense cytoplasm</td>
<td>Relative monomorphism</td>
<td>Infrequently necrotic debris with fibrin</td>
</tr>
<tr>
<td>Lacking course chromatin</td>
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<tr>
<td>Infrequent nucleoli</td>
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</tbody>
</table>

Table 2 Summary of decoy cell (polyomavirocyte) nuclear and cytoplasmic features using 95% ethanol fixation and Papanicolaou staining, with overall presentation and background characteristics.

considerations. This ensures optimal Papanicolaou staining
and cellular transparency, allowing clear visualization of
nuclear detail, thus diagnostic efficacy. Air-drying can
introduce extrinsic artifacts that may alter urothelial cell
morphology, thus exacerbate the risks of cytomorphologic
overlap with misinterpretation.

Because voided urine is typically hypocellular, cyto-
centrifugation is particularly well suited when using low
centrifugal forces and spin times, with particular attention
to ensure immediate fixation. Urine samples ought to be
adequately diluted if hypercellular, with the cytocentrifuge
programmed accordingly to minimize cellular overcrowding
and a false sense of cell clustering. This may occur particu-
larly as cells may be forced together intimately because of
either improper specimen dilution or excessive centrifugal
force coupled with the restrictive small glass slide area upon
which cells are forcefully deposited.

Given the superior cellular preservation typicallyach-
ved through liquid-based monolayer technology, it is
preferential if voided urine specimens have adequate or
hyper-cellularity. Liquid-based methodologies may elimi-
ate air-drying, hence produce exceptional cyto-preparations.

Cell-block sections may also be produced from hyper-
cellular urinary specimens facilitating special staining and
quantitative analyses.

In series studies, the number of decoy cells noted cyto-
logically can correlate with the degree of viral reactivation
and BKVAN, and therefore reflect overall viral load.37-39
Cytologic decoy cell quantification may be a dependable
tool for gauging viral load as 10% to 30% of BKVAN pa-
tients may exhibit decoy cell viruria, whereas 13% may
have PyV viremia.6-8,10,11,28 Note, however, that cytopa-
thology cannot distinguish between BKPyV and JCPyV
infections morphologically.

Appropriate surveillance and modulation of immuno-
suppression is critical and may be facilitated through
cytologic decoy cell quantification. This is particularly
important because only for BKPyV does degree of viruria
 correlate with degree of immunosuppression, and because
PyV reactivation precedes the clinical symptoms of
BKVAN.3-8,10,11,28
awareness of the technical parameters specific to the cytopreparatory modalities used in modern practice is important.

Decoy cell rationale

The obscure cells Papanicolaou described in the Atlas of Exfoliative Cytology (1954) were arguably decoy cells based on their nuclear chromatin patterns and plasmacytoid, elongated cellular shapes, and overall cellular presentation and staining (Fig. 3).

Despite the scientific developments overall, and in keeping with the decoy cell interpretive rationale, any urothelial cells exhibiting cytomorphic features resembling those of polyomavirocytes cannot unequivocally exclude the possibility of high-grade urothelial carcinoma, or co-existence of two pathobiologies in healthy and immunocompromised patients. Essentially, the decoy cell pitfall may be bi-directional; and this may be particularly true in technically suboptimal cytologic cases.

In 1954 Papanicolaou reported the presence of obscure urothelial cells in “cases of malignancy”, and that their distinctive, hence contrasting, features were high N:C ratios and eccentrically located nuclei. But malignant cells arising from high-grade urothelial carcinoma can display such features. Therefore, the dilemma resulting from such cytomorphic overlap merits consideration, particularly as recent studies reported possible polyomavirus involvement in urothelial neoplasia. Certainly, immunohistochemistry and PCR may be useful reflex ancillary analyses to either confirm or disprove the cytologic findings.

Nevertheless, when screening for possible PyV cytopathy, practicing cytologists need to deliberate a range of urothelial pathobiology within a framework of the cytologic findings, patient history, mode of cytopreparation, and adequacy of both fixation and Papanicolaou staining. With careful attention to cytomorphology, interpretations of urinary tract PyV involvement through decoy cell identification and quantification may be made with diagnostic certainty to aid effectively in clinical decision-making.

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