

# Human papovavirus in Papanicolaou smears of urinary sediment detected by transmission electron microscopy

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**SUMMARY** A technique is described whereby inclusion-bearing cells identified by light microscopy in stained smears of urinary sediment were reprocessed for examination in the electron microscope. The nuclei of the abnormal cells were found to contain numerous virus particles, 35 nm in diameter, which morphologically resembled papovaviruses. The technique was applied in this case to identify further the virus producing the cytopathic changes in the Papanicolaou smear. It could be particularly valuable for retrospective studies of mounted cytological or histological material when suitable specimens are no longer available for virological investigation.

A recent development in diagnostic cytology has been the application of the technique to the detection of virus infections in patients whose immunity is impaired by therapy or disease (Bossen *et al.*, 1969; Coleman *et al.*, 1973; Traystman *et al.*, 1976). These patients are susceptible to infection of the urinary tract with cytomegalovirus and human papovavirus, and virus activity may be accompanied by cytopathological changes in the urothelium. Large viral inclusions may form in the nuclei of infected cells which are exfoliated into the urine where they can be detected by light microscopy of stained smears of the urinary sediment. The presence of inclusion-bearing cells in cytological preparations is an indication for further study of the urine by virological methods to confirm the cytological opinion and accurately identify the virus.

In this paper we describe a method whereby smears containing inclusion-bearing cells identified during cytological screening were processed further and embedded in epoxy resin for examination in the electron microscope. The technique of embedding the smear is a modification of that developed by Tun Pe with one of us (JFM) for electron microscopic examination of immunoperoxidase stained sections (Tun Pe, 1975).

## Material and methods

### CYTOLOGICAL SPECIMENS

Six smears were prepared by cytocentrifugation

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(Shandon) from a 20-ml urine sample from a female patient who was receiving a course of cyclophosphamide therapy for nephrotic syndrome. The smears were fixed in ethyl alcohol, stained by the Papanicolaou method, and examined by light microscopy.

Numerous enlarged abnormal urothelial cells, about 35  $\mu$  diameter, were observed in the smears (Figs 1 and 2). The nuclei of most of the cells were hyperchromatic and structureless and had prominent, greatly thickened nuclear membranes. In some cells the nucleus contained a large basophilic intranuclear inclusion which was separated from the nuclear membrane by a clear halo, and the position of these cells in the smear was clearly marked on the coverslip.

### ELECTRON MICROSCOPY

A single inclusion-bearing cell was identified in the Papanicolaou smear of the urine sediment and its position was marked on the under surface of the slide. The slide was immersed in xylol and the coverslip floated off. The smear was taken through graded alcohols to distilled water and post-fixed by flooding the slide for 15 minutes at 4°C with osmium tetroxide in veronal buffer (pH 7.4). After being rinsed in distilled water, the smear was flooded with saturated uranyl acetate for 10 minutes at room temperature, dehydrated in graded alcohols, infiltrated with acetone/epoxy resin mixture, and embedded in an Epon 812 resin mixture (TAAB)

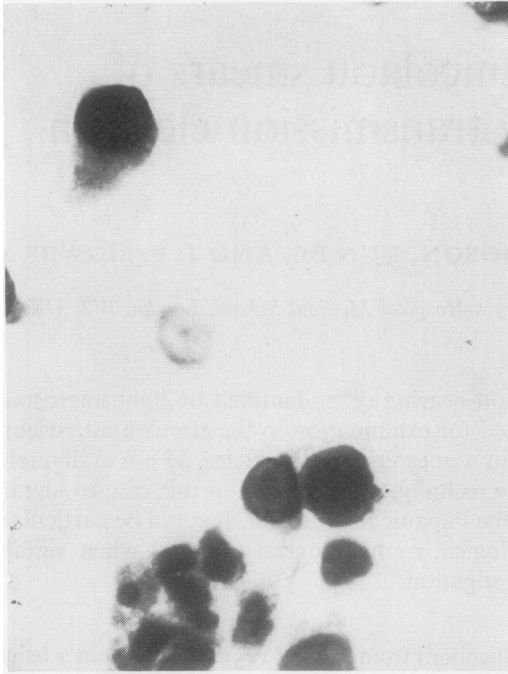


Fig. 1 Smear of urinary sediment showing large abnormal urothelial cells with hyperchromatic structureless nuclei and greatly thickened nuclear membranes. Papanicolaou stain  $\times 770$ .

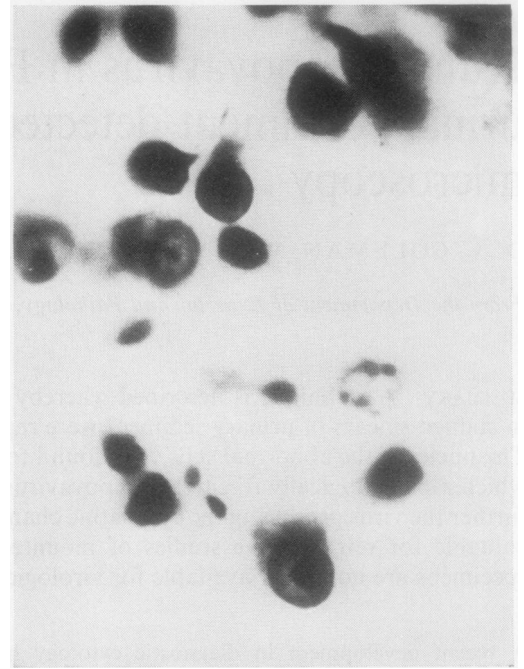


Fig. 2 Smear of urinary sediment showing urothelial cells containing basophilic intranuclear inclusions. Papanicolaou stain  $\times 770$

according to the method of Rowden and Lewis (1974). Care was taken throughout the procedure that the smear did not become air-dried.

The tip of a conical polyethylene embedding capsule (TAAB) was sliced off with a scalpel blade, leaving an aperture approximately 2 mm in diameter. The cut surface was heat polished by being melted against a hot glass slide while held in a plastic former. The smear was covered with resin, the excess removed, and the aperture of the inverted capsule positioned over the marked cell by use of a Perspex holder which kept the capsule firmly in place in very tight contact with the slide. The capsule was filled with resin mixture and the slide and capsule in the holder were incubated at 60°C overnight. The following morning the slide and adherent capsule were removed from the holder and rapidly immersed in liquid nitrogen. The difference in thermal coefficients of expansion of glass and epoxy is such that the slide cleaved off the epoxy cleanly. The resin was removed from the capsule and the single cell from the Papanicolaou smear was identified again in the smear which now occupied the superficial layer of the tip of the epoxy resin block. The resin was trimmed, and ultra thin sections were cut and examined in a Phillips EM 300 microscope.

## Results

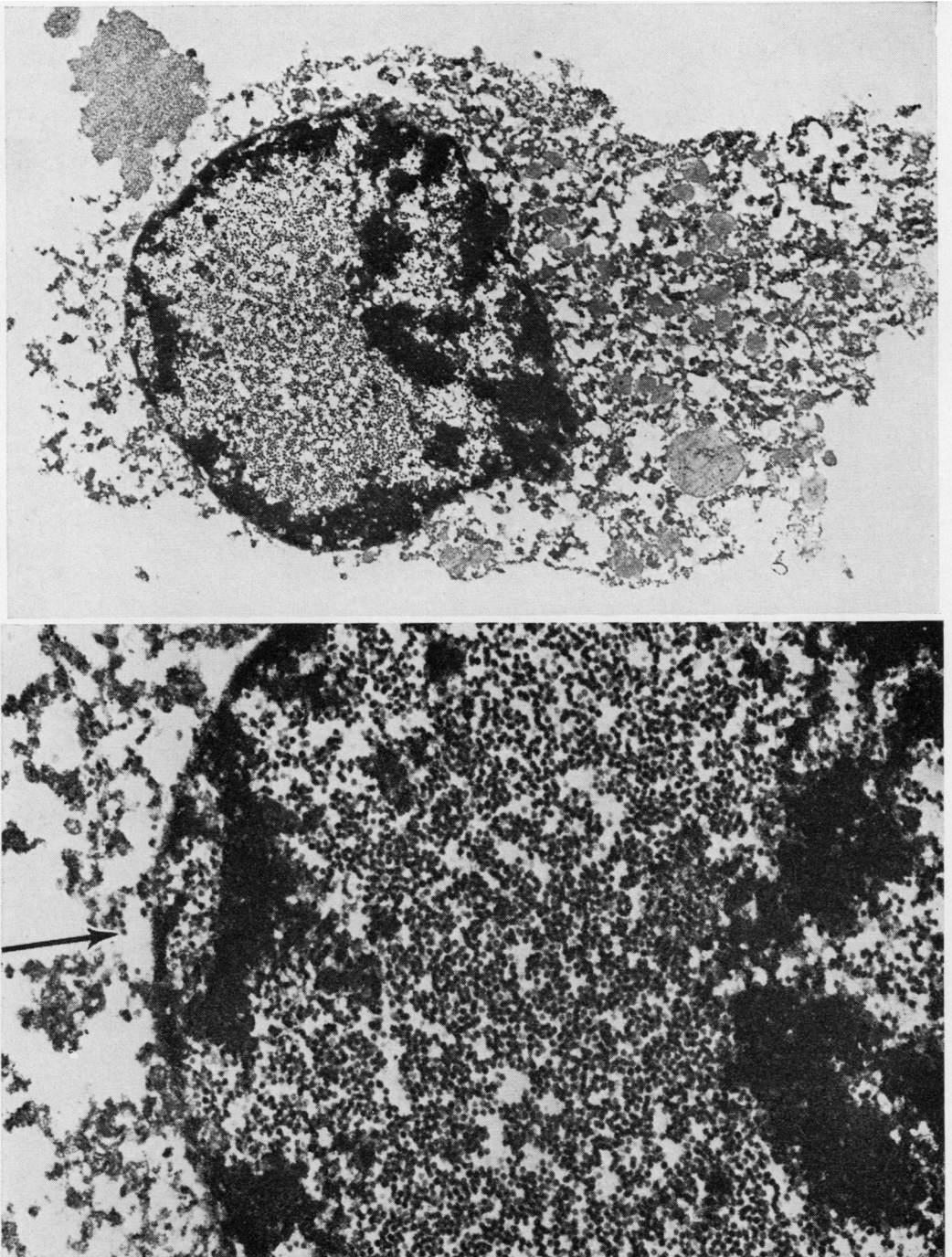
### ELECTRON MICROSCOPY

Cells prepared for electron microscopy were seen to contain large numbers of uniform spherical virus particles in the nucleus (Fig. 3a and b). Many of the virus particles were freely scattered between small clumps of nuclear chromatin; others were assembled in small, loose, crystalline lattices (Fig. 4a and b).

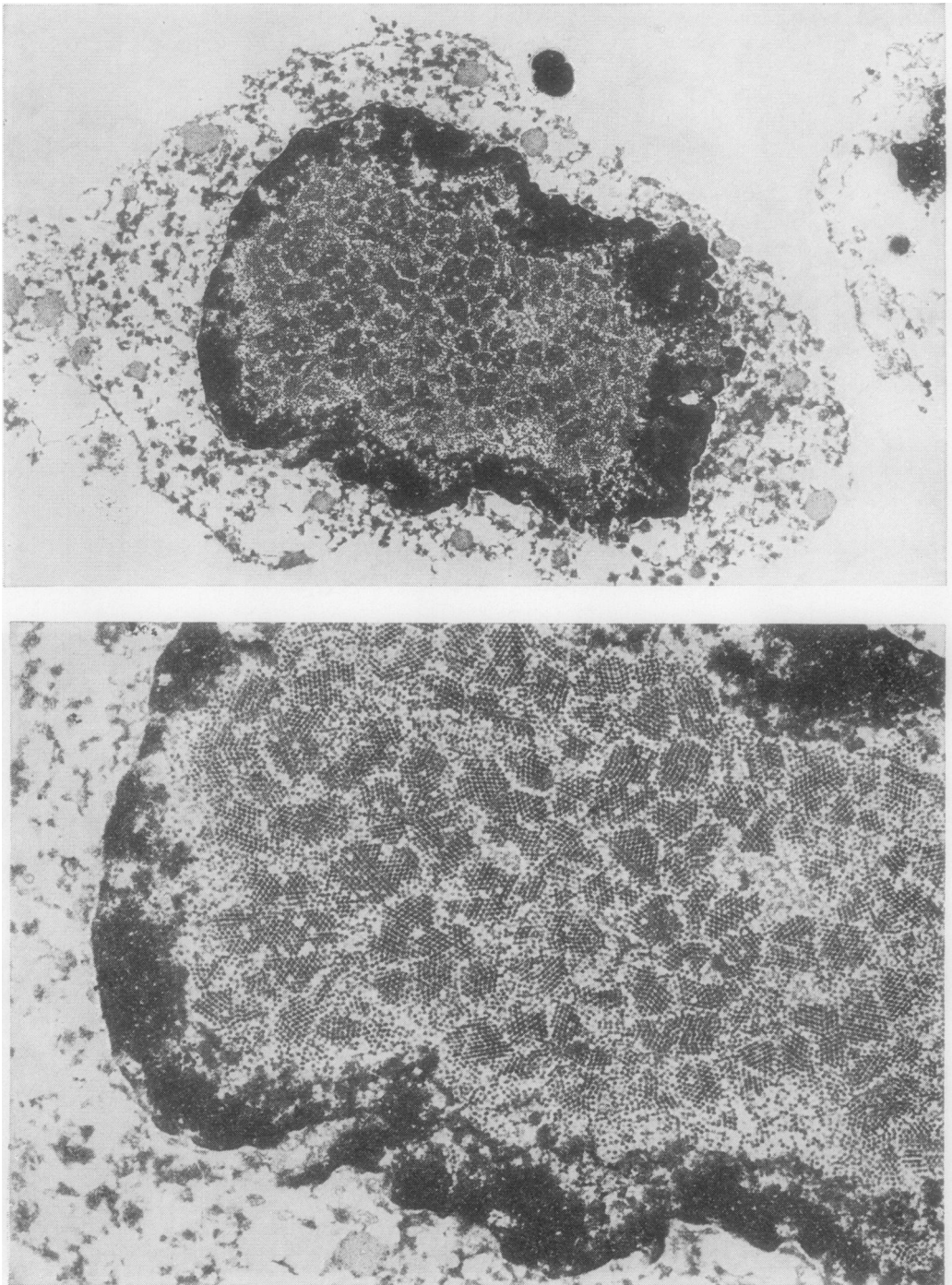
The diameter of the virus particles was found to be on average 35 nm and their morphology in thin section resembled that of the papovaviruses. In cells with an intact nuclear membrane all the virus particles were contained within the nucleus; where the nuclear membrane was ruptured, a few virus particles were observed in the cytoplasm. Although the virus particles were clearly discernible, the fine structure of the nucleus and cytoplasm of the cells prepared in this way was poorly preserved.

### Discussion

The virus particles seen in the electron microscope exhibited features characteristic of the Papovaviridae (Andrewes and Pereira, 1972). The papovaviruses are DNA viruses, with an icosahedral symmetry of



**Fig. 3** (above) *Electron micrograph of inclusion-bearing cell identified by light microscopy of stained smears of the urinary sediment. The nucleus contains numerous virus particles. Uranyl acetate  $\times 14\,000$*   
(below) *Part of the nucleus shown above at higher magnification, showing discrete virus particles, 35 nm diameter, some of which are aligned in short chains. Where the nuclear membrane is ruptured a few particles can be seen in the cytoplasm (arrow).  $\times 32\,000$*



**Fig. 4** (above) *Electron micrograph of inclusion-bearing cell from Papanicolaou smear. The virus particles are assembled in small loose crystalline lattices in the nucleus which appears intact. Uranyl acetate  $\times 14\ 000$*   
(below) *Part of the nucleus shown above at higher magnification.  $\times 24\ 000$*

skew form, which develop in orderly patterns within the nucleus of the host cells where they form dense intranuclear inclusion bodies. The papovaviruses are subdivided into two genera—papillomavirus and polyomavirus, according to the size of the virus particles. Members of the papilloma subgroup have particles 52-55 nm in diameter and those of the polyoma subgroup have particles 40-45 nm in diameter.

The particles in the nuclei have a diameter of 35 nm, which is slightly less than that assigned to members of the polyoma subgroup of the papovaviruses. We attribute this discrepancy to technical manipulation of the smear (particularly alcohol fixation) before reprocessing for electron microscopy. The effect of fixation on particle size can be observed from the studies of ZuRhein and Chou (1965) and Howatson *et al.* (1965). ZuRhein reported polyomavirus particles 33-36 nm in diameter in thin sections of formalin-fixed brain from a case of progressive multifocal leucoencephalopathy (PML), whereas Howatson, studying negatively stained preparations from fresh brain material from PML, found the size of the polyomavirions were on average 41 nm. Apart from the apparent shrinkage of the virus particles, the morphology of the viruses in the nuclei is well preserved, in marked contrast to the fine structure of the cell. It is probable that cellular detail could be improved by fixation of the smears in glutaraldehyde before staining.

The members of the papovavirus group which are known to infect humans include infectious wart virus and two recently identified members of the polyoma subgroup, JC virus and BK virus (Gardner *et al.*, 1971; Padgett *et al.*, 1971). Both JC and BK viruses have been recovered from the urinary tract of patients receiving cytotoxic drugs (Gardner, 1977), and cytological examination of the urine for inclusion-bearing cells has been successfully employed as a method of screening renal allograft recipients for evidence of infection with these viruses (Coleman *et al.*, 1973). Active infection of the urinary tract with BK virus appears to be more common than with JC and may be accompanied by the exfoliation of very numerous inclusion-bearing cells into the urine (over 100 per ml urine).

However, immunosuppressed patients are also susceptible to cytomegalovirus infection, and a clear distinction between this infection and infection with BK or JC viruses cannot be made by light microscopy, especially when only a few inclusion-bearing cells are seen in the urinary smears. Further identification by virus isolation or electron microscopy is essential for accurate diagnosis. Virus isolation is the method of choice for confirming infection with cytomegalovirus as herpesvirions are rarely seen in

the urine by electron microscopy even though cytomegalovirus may have been isolated from the specimen (Coleman *et al.*, 1973). In contrast, virus isolation is not always the most practical or efficient method of detecting BK or JC virus infection as the cell cultures frequently require long periods of incubation if the amount of virus in the specimen is small. Electron microscopy of urinary deposits after centrifugation provides an alternative method of confirming papovavirus infection although the sensitivity of the technique again depends on the concentration of virus in the urine. The threshold of visibility for negatively stained preparations is considered to be  $10^6$  particles per ml urine (Madeley, 1972).

The technique we have described enabled us to identify further the virus causing the cytopathic changes in the Papanicolaou smear. We were able to show that papovavirus particles, 35 nm in diameter, were present in the nuclei of the exfoliated urothelial cells. We suggest that this technique could be applied to distinguish the intranuclear inclusions produced by human papovavirus from those produced by cytomegalovirus or other herpesviruses in mounted cytological or histological material when, as in this case, suitable specimens are no longer available for virological investigation.

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