MICROBIOLOGICA, **13**, 27-34, 1990

ELECTRONMICROSCOPIC DEMONSTRATION OF HPV IN ORAL WARTS

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Received May 8, 1989 *Accepted* July 7, 1989

SUMMARY

Human Papilloma Virus (HPV) has been demonstrated in a series of benign proliferative lesions of the skin and the mucosae. The virus has also been found in verrucous laryngeal carcinoma and carcinomas of the oral cavity and other organs. DNA hybridization techniques have classified, the HPV into 51 types, some of which seem to be associated with specific lesions. In order to study the intracellular distribution of HPV, we performed ultrastructural analysis with the electron microscope on 14 specimens taken from 7 patiens by large excissional biopsy, which had been histologically classified as "fibropapilloma". From each patient specimens were taken from both the clinically evident lesion and the clinically normal surrounding mucosa. The specimens were fixed with glutaraldehyde, washed with cacodylate buffer, post-fixed with potassium ferrocyanide reduced-osmium tetroxide, block stained with uranyl acetate and embedded in EPON 812. The tissues underwent to amylase digestion before the electron microscopic examination. We found a large number of viral particles in both nuclei and cytoplasm, without forming crystal array structures as described typically for the virus of the verruca vulgaris (HPV-2). No significant differences were found between the cells derived from the clinical lesion and those derived from the surrounding mucosa. The passage of viral particles from infected to not yet infected cells through the intercellular space was observed. Of particular interest, we found a high intracytoplasmatic presence of the virus and its clear abundancy in the cells surrounding the clinical lesion.

KEY WORDS *HPV, human papilloma virus, fibropapilloma, electron microscopy, oral warts*

INTRODUCTION

The Human Papilloma Virus (HPV) has been demonstrated in benign proliferative lesions of the skin and the mucosae and has also been detected in certain carcinomas of the larynx, the oral mucosa and the uterine cervix. This virus of the papovaviridae family is characterized by an eicosaedric capsid with a diameter of 55nm and a double DNA helix of about 8000 base pairs, complexed with histones and organized in a nucleosoma. Recent proposals suggest forming a separate taxonomic entity. The virus had been

considered not cultivable *in vitro* and Taichman *et al.*, (1983) only recently succeded in doing so. The virus resides prevalently in the cellular nucleus, but only in cells in carcinomatous degeneration has it been seen integrated in the chromosomic DNA. 51 genotypes of the virus have been demonstrated (Orth, 1986; Nuovo, 198). The virus has an elective tropism for the keratinocytes (Taichman *et al.*, 1983), the fibroblasts do not host the virus (Syrjanen *et al.*, 1986). Extrachromosomal viral DNA replication appears to be confined to the lower third of the epithelial layer in its suprabasal part, in the basal part the virus remains in its latent state. With cell differentiation, the virus passes to an active expression, the production of the capsid and the assembly of complete viral particles occurs prevalently in the stratum granulosum and the stratum corneum cells (Orth *et al.*, 1979). The infection of a squamous epithelium by the HPV virus induces a vascular and epithelial proliferation with specific histological configuration which are an exaggeration of the normal tissue architecture. All layers are represented, but are distorted by the growth of subepithelial capillaries, proliferation of the spinous layer and a characteristic degeneration of the surface cells (Howley, 1983). Cell proliferation in the parabasal cells (acanthosis) and growth of dermal capillaries (papillomatosis) are observerd. The continuing synthesis of DNA by the parabasal and intermediate cells is seen microscopically as hyperchromasia, dyscariosis and retarded maturation of the surface cells. In the superficial layers the final event of viral replication produces a characteristic cytopathic effect known as koilocytosys, given by a degenerative aggregation of the chromatin, nuclear collapse and formation of intracytoplasmatic vacuoli. Koilocytosis has been demonstrated to be a fairly specific marker of HPV infection, primarily in the genital mucosa (Orth *et al.*, 1979; Ferenczy *et al.*, 1981; Lutzner *et al.*, 1982). Six types of koilocytes have been reported (Gross *et al.*, 1982) and can be graded according to Ferenczy, (1981). The specificity of koilocytosis also in oral HPV lesions has been demonstrated recently in a comparison study with immunohistochemical demonstration of HPV and presence of koilocytosis (Madinier *et al.*, 1987).

A direct diagnosis of HPV can be done by immunohistochemistry, *in situ* DNA hybridization (Maitland *et al.*, 1987) and ultrastructural analysis with the electron microscope. Molecular hybridization requires specialized personnel and remains at present restricted to specialized research centers. Histochemistry does not permit a detailed analysis of cellular structure and morphological relationship of the virus with its host cell. Electron microscopy, on the other hand, does not permit viral typing, but by preserving the cellular ultrastructure it allows the intracellular distribution of the virus to be studied. Certain viral genotypes seem to prefer characteristic intracellular arrangements, as the intranuclear crystalline agglomerates of the virus of the verruca vulgaris (Lutzner *et al.*, 1982). For other HPV genotypes this structuring has not been seen and a sparse intranuclear distribution has been described, especially those involved in oral lesions (Lutzner, 1983). There are no descriptions of the virus in the cytoplasma and the relationship with the intracellular structures is often insufficiently displayed (Hills *et al.*, 1979). In the present study we have attempted primarily to analyze the intracellular distribution of HPV in oral fibropapillomatous lesions, and secondly, to assess the distribution of the virus in the surrounding clinically normal mucosa (Table 1).

TABLE 1

HPV genotypes associated with specific Head and Neck lesions

Type	Lesion
1	verruca plantaris
2a-e	verruca vulgaris and plantaris
6a-f	laryngeal papilloma
11a, b	laryngeal papilloma
13a,b, 32	focal epithelial hyperplasia of the mouth (FEH)
30	laryngeal squamocellular carcinoma
40	laryngeal carcinoma

MATERIALS AND METHODS

Fibropapillomtous lesions with a large surrounding margin of clinically intact mucosa were obtained from 7 patients by excisional biopsy. Immediately after excision, these specimens were separated into the clinically evident part and the normal surrounding mucosa and processed separately. The specimens were cut into small pieces and processed for routine histological examination and electron microscopy. For electron microscopy, the specimens were cut into 1 mm thick slices and fixed by immersion in 2.5% glutardaldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 6 h at 4°C. Trough several washings with buffer solution, the specimens were postfixed with 1.5% potassium ferrocyanide-reduced 1% osmium tetroxide for 3 h at 4°C. They were then block stained with ethanolated 1% uranyl acetate, dehydrated through a graded ethanol series and embedded in EPON 812. Thin sections were cut using a diamond knife on a Reichert-Jung Ultracut OmU-4, stained with uranyl acetate and lead citrate and examined with a Hitachi HU-12A electron microscope at 75 kV. After sectioning, some grids with sections were floated on amylase solution to digest glycogen granules in the cells. In addition, isolated HPVs were directly mounted on formvar-coated grids, negatively stained with 1% phosphotungstic acid and examined with the electron microscope as described above. Isolated HPVs were kindly provided by Dr. L. Taichman, Department of Oral Biology and Pathology, State University of New York at Stony Brook, USA (Table 2).

RESULTS

The results of conventional histologic analysis are shown in Table 2. Unfortunately it was not possible to obtain information regarding koilocytosis or other specific cell aspects suggesting HPV infection. In electron microscopy, isolated and negatively stained HPV appeared as round particles (Fig. 1). In all 14 specimen examined, similar round particles of 40-55nm in diameter, compatible with the HP virus (Hills *et al.*, 1979; Nakajima *et al.*, 1985), were observed in the squamous cells. In amylase-treated sections, these electron-dense viral particles were clearly distinguished from digested glycogen granules forming electron-lucent spaces in the cytoplasm (Fig. 2). The viral particles were visible in both the nucleus and the cytoplasm of the cells of the prickle and granular cell layers in the clinically evident lesion as well as those of the surrounding mucosa (Figs. 2, 3, 4). Viral particles were furthermore observed in the extracellular spaces of the granular layer but could rarely be seen in the basal cell layer (Fig. 4, 5). The coexistence of already infected cells and those still without viral particles was also noted (Fig. 5). Viral particles were particularly abundant in the cytoplasm but never formed crystalline agglomerates (Fig. 2, 4).

TABLE 2
Lesions

Number Name	Age	Sex	Clinical aspect	Region	Ligt Microscopy
1 L.P.	51y	M	verrucous, exophytic	left cheek	pseudopolyp
2 V.R.	61y	F	soft, flat	tongue border	fibropapilloma
3 D.V.	37y	M	verrucous, exophytic	velum	acanthosis
4 G.G.	57y	F	exophytic, pedunculated	velum	fibropapilloma
5 A.C.	57y	F	exophytic	velum	fibropapilloma
6 M.S.	42y	M	exophytic	velum	fibropapilloma
7 G.N.	38y	F	verrucous, exophytic	velum	fibropapilloma

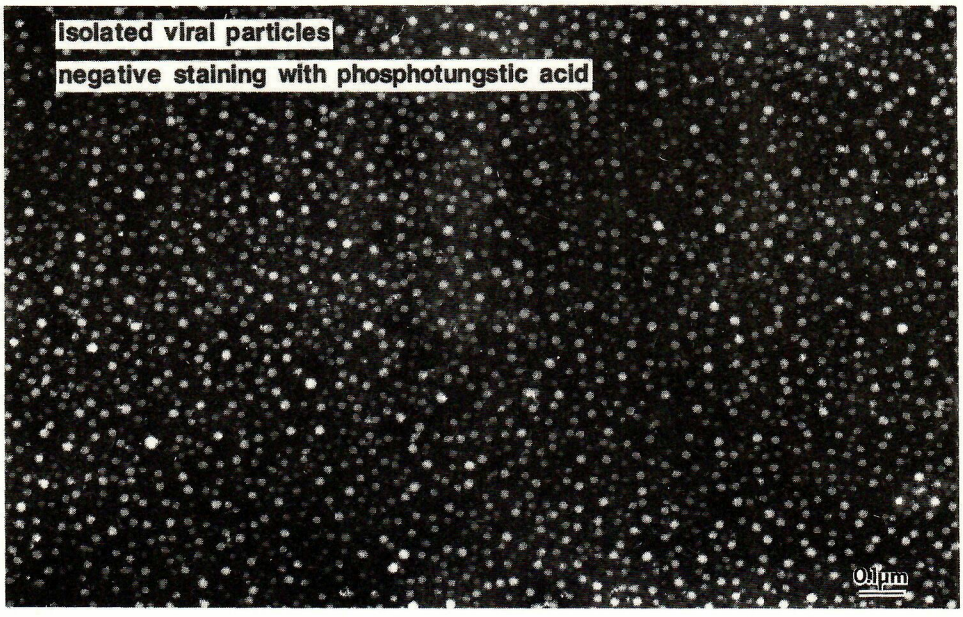

FIGURE 1 - Negative staining of isolated HPV. x 75,000

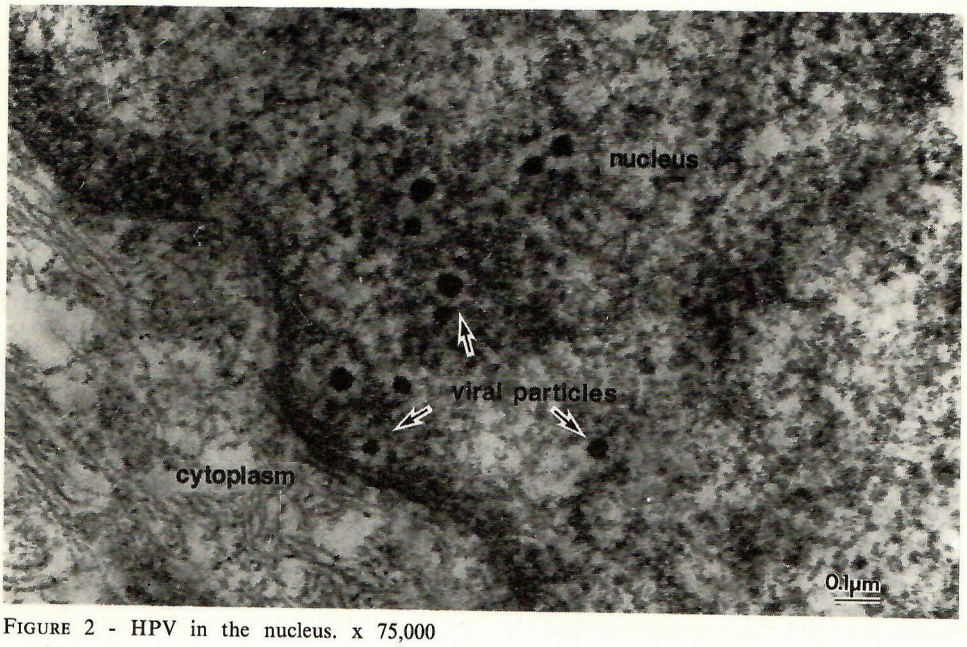

FIGURE 2 - HPV in the nucleus. x 75,000

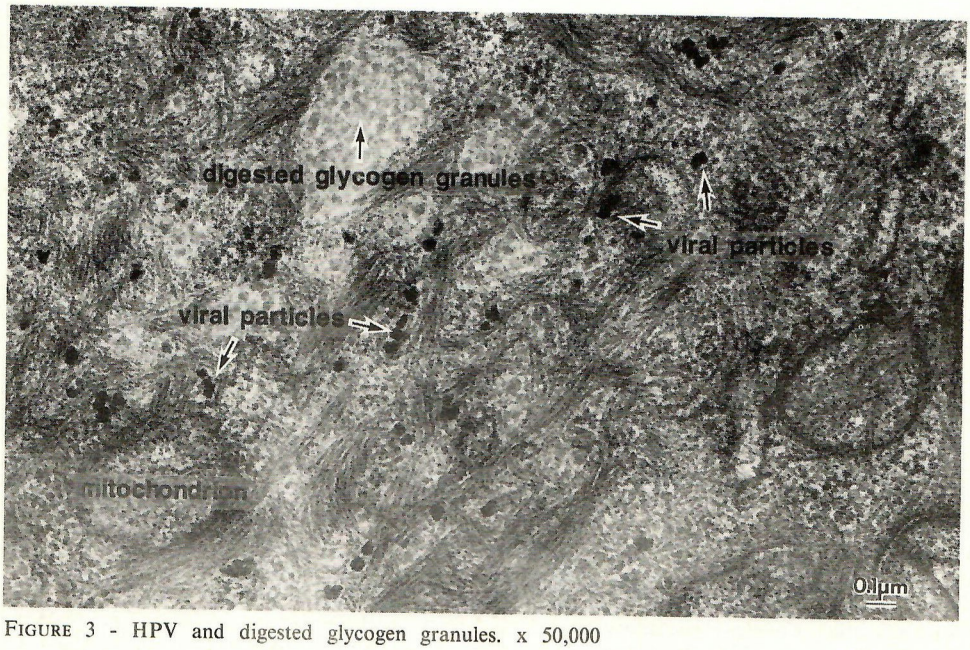

FIGURE 3 - HPV and digested glycogen granules. x 50,000

In several cells derived from the surrounding mucosa, large intracytoplasmatic agglomerates were visible without the evidence of virus in the nucleus.

DISCUSSION

Fibropapillomatous lesions are seen more and more often in the mouth. These lesions have been linked to HPV infection (Beaudon *et al.,* 1987; Rozell *et al.,* 1986; Lookingbill *et al.,* 1987). This virus has recently been at the center of growing scientific interest for two major reasons. First, it has been seen that certain virus types are associated with an increasing number of carcinomas, especially those of the uterine cervix where most work has been done. There is evidence that the HPV virus may also be actively involved in the development of oral carcinomas (Eisenberg *et al.,* 1985; Howley, 1986, Dekmezian *et al.,* 1987). Secondly, the virus is increasingly seen in clinical infections, especially those associated with Human Immunodeficiency virus (HIV).

In the Acquired Immunodeficiency Syndrome (AIDS) infections with virus, bacteria and protozoa are the most frequent, as is the incidence of neoplasms. Convergence of the activity favoring the development of neoplasms of both HPV and HIV viruses, which are both thought to be mainly sexually transmitted, has been suggested, but up to now no association studies of the two viruses in the oral region have been done.

It was our intent to look into the presence and spatial intracellular distribution of the HPV virus in oral lesions and in the surrounding clinically normal mucosa. We were able to demonstrate the virus not only in the cell nucleus, but also in the cytoplasm. The preparation technique preserved the cellular ultrastructure of the keratinized cells well enough to see the passage of viral particles from one cell to another.

FIGURE 4 - Virus in the cytoplasm and the extracellular spaces. x 25,000

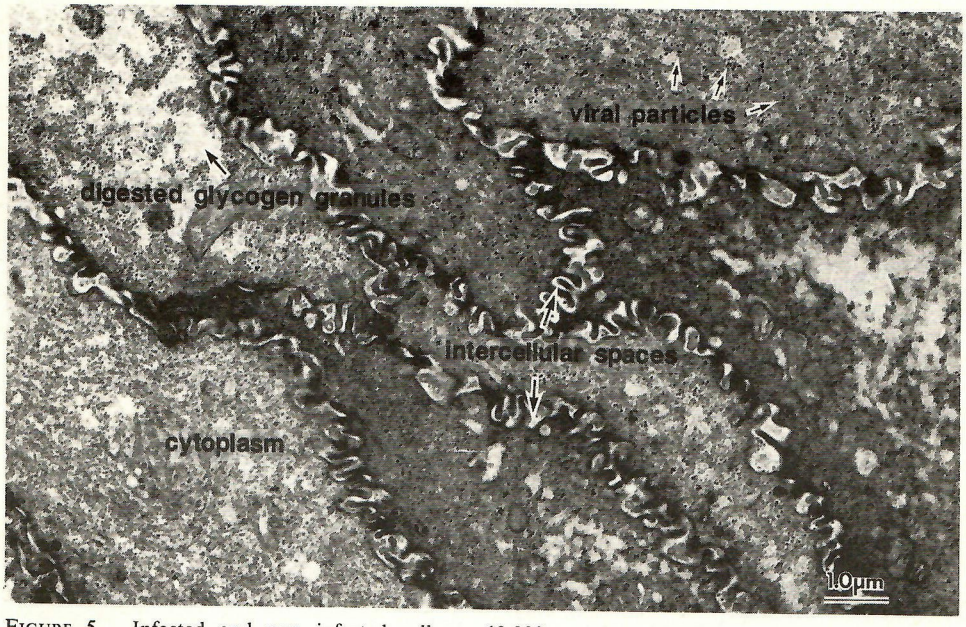

FIGURE 5 - Infected and non infected cells. x 10,000

The viral particles appeared in all specimens in the suprabasal layers, while the basal cells appeared to be free of them. Of special interest is that we were able to demonstrate the presence fo the virus in the clinically normal mucosa which surrounds the lesion. Similarly to the situation in gynaecology, where frequent colposcopic and cytologic controls are generally considered necessary in persons positive for cervical HPV, the possibility of malignant degeneration of the infected squamous cells in the oral region must be taken into account. Frequent controls should be considered after the surgical removal of the fibropapillomatous lesions. The virus remains certainly present in the mucosa, even without expressing its ability to induce macroscopic cell proliferation.

ACKNOWLEDGEMENTS

We thank Dr. L.B. Taichman, Department of Oral Biology and Pathology of the State University of New York at Stony Brook, New York, USA, for giving us his HPV specimen and Dr. K. Barosi, Department of Pharmacology, P.O.C., USSL n. 51, Cremona, for assisting us in the preparation of some of the chemicals.

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