Polyomavirus-induced pilomatricomas in mice: from viral inoculation to tumour development

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Polyomavirus has been used extensively to study tumour induction in mice. Although most neoplasms are well characterized, those arising from hair follicles have been referred to by different names during the last four decades. The purpose of this research was to contribute to a more accurate histological characterization of these tumours as well as to study the viral progression from the onset of infection to the development of neoplasms. Polyomavirus A2 was inoculated into newborn C3H/BiDa mice, and at different time-points (from 5 to 70 days post-inoculation) the mice were sacrificed and studied using histological, immunocytochemical, ultrastructural and virological methods. The fully developed hair follicle tumours consisted of a proliferation of matrix cells that evolved into 'shadow' cells with empty nuclei and finally into amorphous keratin; the tumours were therefore diagnosed as pilomatricomas. Viral VP-1 was observed only in fully differentiated cells and not in proliferating-cell-nuclear-antigen (PCNA)-positive cells in the same tumour. In conclusion, Polyomavirus first replicated in the skin, and then disseminated through the blood and reached the outer sheath of the hair follicles and finally infected matrix cells, leading to the development of pilomatricomas from which infectious virus was isolated.

Key words: Polyoma; pilomatricoma; hair follicle; oncogenesis.

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Polyoma is a small, non-enveloped, double-stranded DNA virus, widely used to study cell transformation *in vitro* and to investigate oncogenesis in mice (1). Its genome encodes three early proteins (LT, MT and ST) and three late-structural proteins (VP-1, VP-2 and VP-3) (1). Although LT is essential for polyomavirus DNA replication and associates with the retinoblastoma protein (2), MT is responsible for most of the mechanisms involved in cell transformation through its interaction with several cell pathways, such as those mediated by pp-60 c-src (3), phosphatidylinositol-3-kinase (4), phosphatase 2 A (5), Shc (6) and 14-3-3 proteins (7). These *in vitro* studies of polyomavirus provided an understanding of the mechanisms involving key cellular regulatory factors in cell transformation. The major capsid protein VP-1 is mainly involved in polyomavirus tropism (8).

After inoculation into newborn mice, some polyomavirus strains induce neoplasms arising from as many as 12 different tissues (9); the neoplasms can be grossly detected a few months later. Thymic epitheliomas, mammary gland adenocarcinomas, salivary gland adenocarcinomas, kidney sarcomas and 'hair follicle

tumours' have been described (9). Although the morphological characteristics of the mammary gland, thymus, salivary gland and kidney neoplasms are well known, the histological characterization of 'hair follicle' tumours is not well developed.

The first description of 'hair follicle' tumours appeared in 1963, shortly after the discovery of polyomavirus (10) and similarities and differences between polyomavirus-induced murine 'hair follicle tumours' and human Calcifying Epithelioma of Malherbe were noted. Two decades later, several key studies described the association of polyomavirus genes with different aspects of tumour profiles in mice, including the 'hair follicle tumours'. In most of these studies, the skin-adnexal neoplasms induced by polyomavirus in mice were described simply as 'hair follicle tumours', without further elaboration (11); (12–14). In others, tumours were diagnosed as 'inverted papillomas' (15), 'epydermoid cysts', 'intradermal unerupted papillomas' or 'cystic trichoepitheliomas' (16). The polyomavirus-mouse system is an important tool for studying the interaction between a viral infection and the induction of neoplasms. Thus, it is important to fully characterize every neoplasm arising from polyomavirus-infected mice.

This study has three specific aims: first, to contribute to the histological characterization of 'hair follicle' tumours; second, to study the viral progression from the moment of infection to the development of the tumours; third, to determine the relationship between polyomavirus replication and cell proliferation in hair follicle neoplasms.

MATERIAL AND METHODS

Virus

We used a highly oncogenic murine polyomavirus strain (A2) obtained from Paolo Amati (University of Rome 'La Sapienza'). Viral stocks were produced by infecting primary baby mouse kidney (BMK) cell cultures prepared from specific pathogen-free Balb/c mice (obtained from the bioterium of the University of La Plata, School of Veterinary Medicine, Argentina). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Carlsbad, CA, USA) and 10% foetal calf serum (FCS) (GIBCO, Carlsbad, CA, USA), incubated at 37 °C in a 5%

CO_2 atmosphere. Virus stocks were prepared by freezing and thawing the infected cell cultures three times after complete cytopathical effect was observed, titrated by plaquing under agar and maintained frozen until use. The same procedure was performed for non-infected BMK cells, for using as mock inocula.

Animals

Pregnant C3H/Bittner-Dawe (C3H/BiDa) mice were housed in individual boxes maintained at room temperature and fed on pellets *ad libitum*. Newborn males or females (24 h-old) were subcutaneously inoculated either with polyomavirus or with the supernatant of uninfected BMK cells. The rules of the University of Buenos Aires for Experimental Animals Welfare were observed during the experiments.

Cells

NIH-3T3 cells were cultivated on glass cover slips stored in 60-mm plastic petri dishes at 37 °C and supplied with DMEM plus 10% FCS in a 5% CO_2 atmosphere.

Indirect immunofluorescence (IFI)

NIH-3T3 cell cultures previously adsorbed with tissue extracts (obtained from tumours or from normal, uninfected tissues) were fixed with methanol at room temperature for 20 min and washed with phosphate buffer saline (PBS), pH 7.6. After blocking with 5% normal goat serum (GIBCO, Carlsbad, CA, USA) in PBS, the cell cultures were treated with primary rabbit polyclonal anti-VP-1 serum (obtained from Thomas Benjamin, Harvard Medical School), diluted to 1:500 in PBS, for 20 min. Then, rhodamine-conjugated goat anti-rabbit serum (Sigma-Aldrich, St. Louis, MO, USA) diluted to 1:100 in PBS was added for 20 min. Extensive washings with PBS were performed between every procedure. Coverslips were mounted on glass slides using 50% glycerol – 50% PBS, and observed using a Zeiss immunofluorescence microscope with epi-illumination.

Protein electrophoresis and western blotting

Protein extracts were prepared from frozen tumours and from normal skin tissues. Tissues were Dounce-homogenized in cold extraction buffer consisting of 1% NP-40 and 0.1% sodium dodecyl sulphate (SDS) – Tris-buffered saline with protease inhibitors: aprotinin, 1 μg/mL, leupeptin, 1 μg/mL and pepstatin, 1 μg/mL, (Sigma-Aldrich). Protein concentrations were adjusted to 1 μg/μL using the Bradford method and the extracts were resolved in 12%

SDS-acrylamide gels. After blotting onto a nitrocellulose membrane as described elsewhere, polyomavirus VP-1 was detected using the primary rabbit polyclonal anti VP-1 serum as described in the previous section, diluted to 1:10 000 and a horseradish peroxidase-labelled goat anti-rabbit serum diluted to 1:10 000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Enhanced chemiluminescence (NEN) was employed to develop the exposed Kodak X-Omat AR film.

Virus isolation from tumours

Hair follicle tumours and normal skin tissues were homogenized and protein concentrations were adjusted to 100 µg/mL in PBS. Then, tissue extracts were adsorbed onto NIH-3T3 cell monolayers for 1 h and washed with PBS. After incubation at 37 °C for 24 h, VP-1 was detected by indirect immunofluorescence. Simultaneously, viral titration was performed in NIH-3T3 cells by plaquing under agar.

Viraemia detection

Heparinised blood samples were obtained immediately after animals were sacrificed and were stored at −20 °C until use. The dot-blot method was employed onto nitrocellulose membranes to detect the presence of viral VP-1 in each sample.

Histology

Tissues were fixed overnight in Bouin fluid, dehydrated in ethanol at 96% and 100%, clarified in xylene and routinely embedded in paraffin. Slides were stained with haematoxylin-eosin, as described elsewhere.

Immunocytochemistry

The peroxidase-antiperoxidase (PAP) technique was employed to detect polyomavirus VP-1 as previously described (17, 18) by using the primary serum mentioned in the previous section or proliferative-cell-nuclear-antigen (PCNA), using rabbit polyclonal serum (Santa Cruz Biotechnology) in paraffin-embedded slides. Endogen peroxidase was blocked with 5% H_2O_2 diluted in methanol for 30 min. Slides were then treated with 0.05 N Tris-Cl (pH 7.6) and blocking was performed with 5% normal goat serum (GIBCO, Carlsbad, CA, USA) in Tris-CL (pH 7.6) at 37 °C for 20 min. The first serum was applied overnight at 4 °C. The rabbit polyclonal anti-VP-1 serum was diluted to 1:500 and anti-PCNA was diluted to 1:100. The secondary antibody was goat anti-rabbit immunoglobulin, diluted to 1:50 (DAKO, Carpinteria, CA, USA) and the tertiary antibody was the labelled PAP serum prepared in rabbit (DAKO), diluted to 1:250. Extensive washings with 0.05N Tris-Cl were performed between the applications of each serum. Slides were developed under microscopy control using 3-3′diaminobenzydine (FLUKA, Sigma-Aldrich) in 0.05N Tris-Cl and 5% of H_2O_2 as the substrate. Slides were slightly counterstained with haematoxylin and mounted as usual.

Transmission electron microscopy

Immediately after mice were sacrificed, tissues were minced in a drop of fixative composed of 4% formaldehyde freshly prepared from paraformaldehyde and 1% glutaraldehyde in PBS, pH 7.4 and fixed for 45 min, and then post-fixed in 1% osmium tetroxide in PBS for 1 h and routinely processed for Durcupan ACM (FLUKA, Sigma-Aldrich) embedding. Slides were obtained with glass knives and stained with uranyl acetate and lead citrate. Samples were observed using a Zeiss EM-109-T transmission electron microscope (TEM) at 80 kV.

RESULTS

Thirty mice (18 males and 12 females) were subcutaneously inoculated with 5×10^5 plaque forming units (pfu) of polyomavirus A2 contained in 0.05 mL supernatant of BMK-infected cells; 18 mice were injected with the same volume of supernatant of mock-infected BMK cells as a negative control. Inoculations were performed in the right flank, and each animal was clinically examined twice a week until tumours were detected. At 5, 10, 25, 40 and 55 days post inoculation (PI), five animals were sacrificed per time-point; the five remaining animals were killed when fully developed neoplasms were clinically observed, approximately 70 days PI. Complete necropsy was performed on each animal. Blood samples were obtained under sterile conditions and frozen. Samples of every tissue were fixed in Bouin fluid, paraffin-embedded and used for histological and immunocytochemical observations and descriptions. We used immunocytochemistry to detect polyomavirus VP-1 and not DNA *in situ* hybridization as the presence of a late, structural viral protein is more indicative of viral replication than the presence of the whole viral genome (15, 18). Samples of subcutaneous tumours were

immediately minced and processed for ultra-structural studies, as described in the previous section. When visible to the naked eye, subcutaneous tumours were cut through the middle and immediately frozen until later examination for infectious viruses.

At 5 days PI, the only noticeable finding was the intranuclear immunolabelling of VP-1 in dermal skin fibroblasts (Fig. 1), mainly near the area of inoculation. Starting at day 10 PI, VP-1 could be seen in cells belonging to the external sheath of hair follicles (Fig. 2A) disseminating in different areas of the skin, not limited to the site of infection. To guarantee the specificity of labelling, adjacent slides were treated with normal rabbit serum as the primary antibody in each experiment. Moreover, samples of skin belonging to mice inoculated with the supernatant of uninfected BMK cells and other skin samples from normal mice were also used for immunocytochemistry against VP-1. In all cases, the results of labelling were negative. Viraemia reached a spike at day 10 PI (data not shown). Starting at 20 days PI, the proliferation of matrix cells thickened the hair follicles of infected mice, compared with the size and structure of hair follicles in mock-infected control
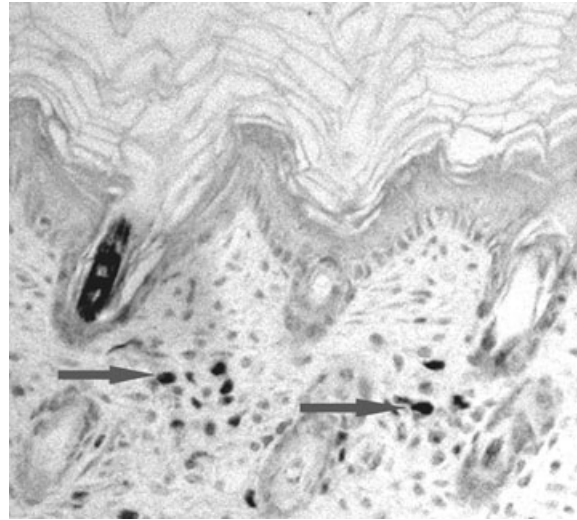

Fig. 1. Detection of polyomavirus VP-1 at the inoculation site of the skin, 5 days post-infection (PI). Several dermal connective cells clearly show intranuclear VP-1 labelling (arrows). The slide has been counterstained with haematoxylin (×140).

mice. At this time-point, tumours were solid masses and VP-1 immunolabelling was seen in matrix cells located in the middle of the hair cortex (Fig. 2B). Fully developed microscopic
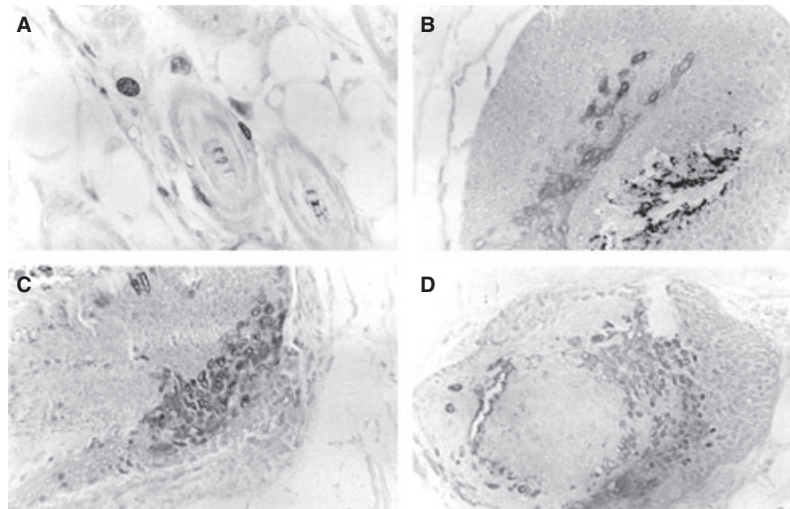

Fig. 2. Progression of polyomavirus infection. The presence of polyomavirus VP-1 (dark labelling) can be observed in cells of the external sheath of a hair follicle at 10 days PI; (A) dermal mesenchymal cell also shows VP-1 labelling (panel A). At 20 days PI (B) and 40 days PI (C), VP-1 was detected in the inner matrix cells of the hair follicles. A more panoramic image of a small, but fully developed pilomatricoma can be observed in panel (D). Note that VP-1 is present in the most differentiated matrix cells, some shadow cells and on the border of the amorphous keratin content only. The slides were slightly counterstained with haematoxylin. Original magnification: (A–C), (×140); (D), (×70).
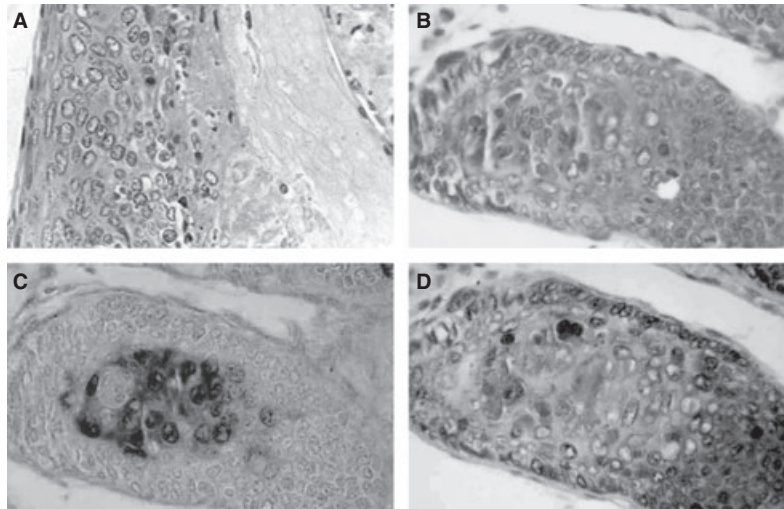
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Fig. 3. Histological image of a fully developed pilomatricoma (A) From left to right, note the differentiation of matrix cells into 'shadow' cells and then into amorphous keratin (haematoxylin-eosin; ×280). Panels (B, C) and (D) show an early stage development of pilomatricoma. Serial slides were taken from the same paraffin-embedded block. In (B), proliferation and maturation of matrix cells can be observed from right to left (haematoxylin-eosin staining). In panel (C), VP-1 (dark labelling) can be detected in the centre of the tumour only, which is composed of differentiated matrix cells. In panel (D), proliferating-cell-nuclear-antigen (PCNA) positive cells (dark labelling) can be observed in the most peripheral matrix cells only. Note that the cells containing VP-1 do not show PCNA and vice versa. Original magnification of panels (B, C) and (D): (×280); panels c and d were slightly counterstained with haematoxylin.

tumours were observed as early as 40 days PI (Fig. 2C). Pilomatricomas were present all over the inner part of the skin of the whole animal and not only limited to the right flank infection site. Each tumour exhibited amorphous keratin content (Fig. 2D). Approximately at 70 days PI, subcutaneous neoplasms could be clinically observed and, at necropsy, they were uncountable, 2–6 mm thick, rounded, brownish, located all over the skin without infiltrating the epidermis; cut surfaces showed an amorphous inner content. The histology of these tumours was characterized by proliferation of matrix cells undergoing differentiation towards 'shadow' cells (with empty nuclei) and finally to keratin debris. The histological image (Fig. 3A) shows a pilomatricoma. In fully developed pilomatricomas, polyomavirus VP-1 was located in the most differentiated layer of the matrix cells, that is, in cells located in the inner part of the tumour and also in 'shadow' cells and in the amorphous keratin contained in the centre of the cystic tumours. To confirm that viral VP-1 was only detected in terminally differentiated matrix cells and not in proliferating cells, serial adjacent slides taken from the same paraffin-embedded blocks were prepared and immunolabelled with primary serum anti-PCNA. As observed in Fig. 3B–D, only the most differentiated PCNA-negative cells contained VP-1. Ultrastructural studies further confirmed this observation; 45 nm, non-enveloped, rounded viral particles were observed in the nucleus and cytoplasm of the most differentiated cells, as well as in the amorphous material (Fig. 4). Infectious virus could be isolated from every single polyomavirus-induced pilomatricoma with an average titre of 10^4 pfu per 100 mg of tissue protein; this was confirmed using IFI after culturing in NIH-3T3 cells and using western blotting (Fig. 5). Interestingly, only pilomatricomas were developed in the skin of polyomavirus-infected mice and no other skin or mucosal neoplasms were observed grossly or at the histology level. Mock-infected mice developed no neoplasms and no VP-1 labelling was detected in their tissues at any time-point. Polyomavirus was also not isolated from any of the control animals.
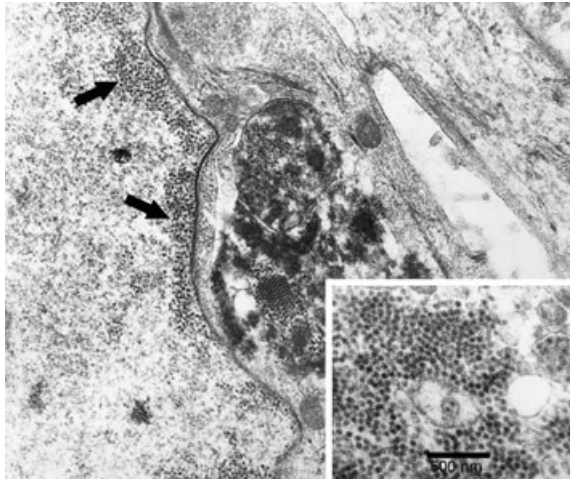
Fig. 4. Ultrastructural image of a well differentiated matrix cell in a pilomatricoma. The 45 nm polyomavirus particles can be observed under the nuclear membrane (arrows) and at higher magnification in the insert.
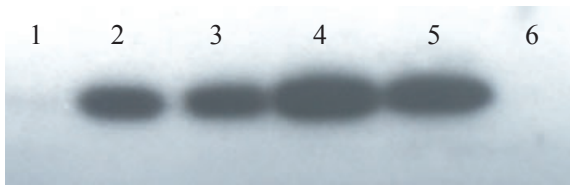

Fig. 5. Detection of VP-1 in pilomatricomas using western blotting. In lane 1, an extract obtained from normal skin of a mock-infected mouse was used as negative control. Lanes 2–4 show the presence of polyomavirus VP-1 in the extracts of three different pilomatricomas. In lane 5, VP-1 can be detected in a diluted stock of polyomavirus, prepared in cultures of baby mouse kidney cells (BMK) and used as positive control. In lane 6, the supernatant of mock-infected cultures of BMK was employed as an internal control and does not contain VP-1. These results further confirm the specificity of the labellings of VP-1 in paraffin-embedded slides, as well as the presence of infectious polyomavirus in pilomatricomas, except for tissues of mock-infected mice.

DISCUSSION

This study investigated 'hair follicle' tumours induced by polyomavirus in mice to contribute to better understanding and characterization of these neoplasms and the relationship between viral infection and tumour induction in this experimental model. Hair follicle tumours were composed of proliferating matrix cells with terminal differentiation towards 'shadow' cells and, finally, amorphous keratin. This histological evidence clearly identifies the tumours as pilomatricomas (19). Although not very common, pilomatricomas are the most frequent benign hair follicle neoplasms in humans, especially in children (20) and patients over 60 years old, and sometimes, they appear as multiple tumours (21). Chromosomal (22, 23) and biochemical (24) disturbances have been described in these neoplasms, but the pathogenesis of these tumours is unknown due to the lack of an appropriate experimental model, leaving aside the spontaneous description of isolated cases in dogs (25). It is often difficult to precisely name skin adnexal tumours, but our findings clarify the histogenesis of these neoplasms, and allow this experimental model to be applied in future investigations. The matrix cell origin of pilomatricomas sets these neoplasms apart from other adnexal-skin tumours.

Patterns of viral infection in polyomavirus-induced pilomatricoma can be divided into three phases. Approximately 5 days PI, viral VP-1 is detected in dermal fibroblasts and mesenchymal cells. This can be understood in terms of local viral replication, mainly at the inoculation site. Later on, viral structural antigens are found in the external sheaths of hair follicles disseminated all over the skin. This observation correlates with a spike in detectable viraemia, suggesting that polyomavirus gained access to the hair follicles after its dissemination through the blood. We were not able to assess if the vessels involved in the passage of the virus from the blood to the external hair follicle sheath are located in the papillae or belong to the capillaries of the superficial dermal plexus. After this initial replication, VP-1 is observed in matrix cells simultaneously with thickening of the hair follicle wall. However, even in the early stages, viral antigens are located in the nuclei of the inner matrix cells, but not in the peripheral cells. Finally, as the neoplasms are fully developed, the structural viral antigens are detected only in the most differentiated, PCNA-negative, matrix cells, in the 'shadow' cells, and in the amorphous keratin that composes the inner parts of the tumours. This sequence of events was observed in every single pilomatricoma and was a consistent and coherent phenomenon. Taken

together, these results suggest that polyomavirus reaches the hair follicles from blood and infects them via the most external hair follicle structure, the outer root sheath; the virus then infects the matrix cells and, somehow, the viral expression of late, structural proteins, progresses simultaneously with the differentiation of matrix cells into terminal, non-nucleated 'shadow' cells and keratin. The presence of VP-1 in these mature cells is not merely the expression of the 'late' part of the polyomavirus genome, as we have described in thymomas (18), but is a consequence of viral encapsidation. This implies that complete viral particles are present in these tumours, as was confirmed by ultrastructural observations and infectious virus isolation *in vitro*. The progression of tumour development in polyomavirus-induced pilomatricoma strongly resembles, but is not identical to that described in human papillomavirus (HPV) infections. As in HPV, the polyomavirus only encapsidates in fully differentiated epidermal cells; however, the polyomavirus first transforms the matrix cells and induces their proliferation and encapsidation occurs only at the end of the process when infectious viral particles are present. This pattern was observed in every single pilomatricoma developed by polyomavirus in mice; therefore, it cannot be considered an isolated phenomenon.

Mice used in the experiments described here also developed other tumours in different organs, as described in previous studies; however, the description of these tumours is beyond the scope of this study. Nevertheless, it is remarkable that polyomavirus maturation, together with epidermal cells differentiation, could be observed exclusively in pilomatricomas, and not in other tumours (data not shown) and no other skin or adnexal tumours were observed, with the exception of pilomatricomas. Taking into consideration that mice were infected with the same viral stock and developed tumours with quite different patterns and processes of viral encapsidation, it is possible that polyomavirus interacts with different cell regulators, depending on the tissue they infect. This means that it is the cell, and not the virus, that determines the pattern of infection and governs the complex relationship between viral replication and tumour induction.

In conclusion, lines of evidence presented here show that 'hair follicle' tumours induced by polyomavirus in mice are, in fact, pilomatricomas and demonstrate that in these pilomatricomas, viral replication takes place in the more differentiated, non-proliferating cells. We also describe a possible mechanism of viral dissemination from the inoculation site until the development of pilomatricomas. Furthermore, we suggest that it is the type of the infected cell that governs the process of polyomavirus infection and transformation, rather than the virus itself.

The recent discovery of a novel human polyomavirus in Merkel cell carcinomas of the skin (26, 27) strongly encourages the study of polyomavirus associated with other human skin neoplasms. We hope the histological characterization of 'hair follicle tumours' induced by polyomavirus in mice as pilomatricomas described here will lead to use this experimental model to perform further studies in the biochemical and molecular mechanisms involved in hair follicle neoplasms pathogenesis.

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