Laboratory Production In Vivo of Infectious Human Papillomavirus Type 11

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Human papillomaviruses (HPV) induce among patients natural lesions which produce small amounts of virus. Infection of human cell cultures does not lead to the multiplication of virus, which also does not replicate in experimental animals. We have developed a unique system for the laboratory production of HPV type 11 (HPV-11). Fragments of human neonatal foreskin were infected with an extract of naturally occurring human vulvar condylomata and grafted beneath the renal capsule of athymic mice. Later (3 to 5 months), condylomatous cysts developed from those grafts. Nuclei of koilocytotic cells contained large amounts of capsid antigen and intranuclear virions. The experimentally induced condylomata were homogenized, and the virions were extracted and used to infect another generation of human foreskin grafts in athymic mice. The HPV-11 DNA content and infectivity of the natural and experimental condylomata were similar. Extracts of experimental condylomata were subjected to differential ultracentrifugation and sedimentation in CsCl density gradients. A single, opalescent band was visible at a density of 1.34 g/ml. It contained HPV virions with HPV-11 DNA. This report is the first demonstration of the laboratory production of an HPV.

One of the major obstacles to human papillomavirus (HPV) research is the inability to produce in the laboratory sufficient amounts of intact virions that can be used to infect cells or elicit antibody production in experimental animals. HPV shows incomplete and transient replication in human epidermal cell cultures (9), and there are no satisfactory laboratory animal substitutes. HPV-induced lesions often contain demonstrable amounts of episomal viral DNA, but only rarely do they produce large quantities of complete virions. Highly keratinized warts such as plantar and common warts may be an exception to this generalization, since they frequently contain large quantities of virions (4). Factors responsible for the wart lesion properties that determine the amount of virus produced are not known. Also, because of the lack of an in vitro system for virus production, we do not know how virion expression is regulated for any HPV.

We have recently described a system which permits HPV to infect and morphologically transform human tissues in vivo under controlled laboratory conditions (7, 8). Fragments of human epithelium were infected with HPV type 11 (HPV-11) and grafted beneath the renal capsule of athymic mice. After 3 to 5 months, the tissues developed into experimental lesions identical to naturally occurring lesions of patients. Experimental condylomata of the cervix and skin often contained abundant koilocytotic cells which strongly reacted with antiserum against the HPV group-specific antigen. The purpose of the present investigation was to test the hypothesis that experimental condylomata might constitute a useful source of infectious virus and purified virions.

Athymic mice (*nu/nu* on a Swiss background) were purchased from Mammalian Genetics, National Cancer Institute, Bethesda, Md. Mice were placed in flexible film isolators supplied with sterile air, food, and water.

Human foreskins were obtained from neonatal circumcisions and were free of gross or microscopic lesions. Split-thickness skin grafts were cut with a sterile single-edged razor blade or a scalpel. Grafts were held in minimum essential medium with only gentamicin (40 mg/ml) as a supplement and were trimmed to dimensions of 2 by 2 by 0.5 mm. Grafts were then rinsed and incubated for 1 h at 37°C in 0.1 ml of one of two treatments: phosphate-buffered saline (PBS) or a PBS extract of natural vulvar condylomata acuminata (designated CA 1). Details of the preparation of the natural condylomata extract have been previously described (8). The extract contained HPV-11 DNA as shown by Southern blot analysis and was free of detectable HPV-1, -6, -16, and -18 DNA (7). Mice were anesthetized, and the kidneys were exposed through dorsal incisions. The renal capsule was nicked, and a skin graft was inserted with toothless forceps as described elsewhere (7). Skin incisions were closed with wound clips.

After 3 to 5 months of growth, the mice were killed by cervical dislocation. Foreskin grafts transformed by HPV-11 often produced thin-wall condylomatous cysts 1 to 2 cm in diameter. They were incised, and the keratin cores were removed for virus extraction. Conventional paraffin sections were prepared from the condylomatous cyst walls and stained with hematoxylin and eosin and by the immunoperoxidase method for the demonstration of the group-specific antigen of the papillomaviruses (5). These sections showed typical condylomatous changes, consisting of koilocytosis, hyperplasia, and hyperkeratosis (7). Nuclei positive for group-specific antigen were often abundant (7). For electron microscopy, grossly papillomatous regions of the interior cyst wall were identified with a dissecting microscope and 1-mm cubes were excised, fixed, and sectioned. Tissue fragments were selected from a papillomatous region and placed in a fixative (3.7% Formalin, 1% glutaraldehyde in 0.1 M cacodylate buffer). Tissues were postfixed in 1% osmium

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FIG. 1. Thin section of plastic-embedded HPV-11-infected foreskin graft. Almost all available space within the heterochromatin is occupied by dense arrays of osmiophilic HPV-11. The inset shows an enlargement of a region with HPV-11 particles.

tetroxide in buffer for 1.5 h, placed into alcohol-acetone before uranyl acetate staining en bloc, then dehydrated through graded alcohols, and embedded in polybed medium. Sections (50 to 70 nm thick) were cut on an ultramicrotome (Ivan Sorvall, Inc., Norwalk, Conn.), collected on 200-mesh copper grids, stained with lead citrate, and then examined with a Phillips 200 electron microscope. Many of the koilocytotic nuclei contained intranuclear arrays of virions that were internal to the heterochromatic nuclear margin (Fig. 1). The intranuclear location of abundant virions strongly argued against the possibility that this was input virus. The original inoculum ought to be randomly distributed and is most unlikely to persist in the koilocytotic layers of an epithelial population which has renewed itself many-fold over several months.

In a previous study (7), we demonstrated by Southern blot hybridization that experimental condylomata contained HPV-11 DNA and were negative for HPV-1, -6, -16, and -18 DNA. We have not directly compared experimental and natural condylomata for the relative amounts of hybridizable HPV-11 DNA and biological infectivity for a new generation of human foreskin grafts. For this purpose, approximately 7.8 g of four experimentally produced condylomata (each about 1.5 cm in diameter) was pooled, homogenized, and extracted with PBS and clarified by low-speed centrifugation. This extract of experimental condylomata was designated CA 2. The HPV-11 DNA content of naturally occurring (CA 1) and experimentally induced (CA 2) condylomata was compared. The direct comparison of the amount of HPV-11 DNA in CA 1 with that in CA 2 is valid, since the ratio of condylomatous mass to solvent was the same in both cases. A 50- μ l volume of serial 1:2 dilutions of extracts was spotted onto cellulose nitrate filter paper, treated with alkali (3), and hybridized under stringent conditions to a 32 P-labeled HPV-11 probe (7, 8). The reactivity of CA 1 DNA had disappeared at a dilution of 1:16, whereas CA 2 DNA was first negative at a dilution of 1:64 (Fig. 2). Thus, both the experimentally induced and the natural condylomata contained similar amounts of HPV-11 DNA.

The CA 2 condylomatous extract also was used to infect fresh foreskin grafts. We conducted three independent ex-

FIG. 2. Dot blot hybridization with 32 P-labeled HPV-11 probe. The relative HPV-11 DNA contents of serial dilutions of natural (CA 1) and experimental (CA 2) condylomata are compared. As a positive control, serial dilutions of 0.5 μ g of HPV-11 DNA in plasmid pML2 were included in the bottom strip.

periments to determine the infectivity of laboratory-produced HPV-11. In experiment 1, a total of 32 CA 2-infected grafts were placed beneath the renal capsules of athymic mice. After 3 months of growth, the mice were killed and the grafts were examined. Although 10 grafts did not take, 16 (73%) of the 22 remaining were condylomatous. Experiment 2 demonstrated that 36 (95%) of 38 foreskin grafts were transformed by CA2, forming condylomatous cysts averaging 1 cm in diameter at 4 months after infection. These proportions of transformed grafts were similar to those which we found after infection with the CA 1 extract of naturally occurring vulvar condylomata (7). In experiment 3, we compared serial dilutions (to the base 10) of extracts of both CA 1 and CA 2 condylomata. The results demonstrated that both preparations were highly infectious when undiluted or diluted to 10^{-1} or 10^{-2} (Table 1). Higher dilutions were negative. The recovery from the grafts of virus that was infectious at a dilution of 10^{-2} after 3 to 4 months beyond the initial infection is strong evidence for HPV replication in the infected grafts.

A small sample of the CA 2 extract was centrifuged in a CsCl gradient. We used blot hybridization to identify the position of HPV-11 DNA. Fractions (0.25 ml) were collected from the bottom of the tube, and 50 μ l of each was spotted on cellulose nitrate paper. The fractions were subjected to alkali to denature the protein and render the DNA single stranded (3). The samples were hybridized with a 32 P nick-translated HPV-11 probe under stringent conditions, as previously described (7). After autoradiography, paper areas corresponding to the fractions were cut out and counted in a liquid scintillation counter. The results (Fig. 3) indicated that all of the HPV-11 DNA was present at a gradient density of 1.34 g/ml. This corresponds to the expected density for HPV virions and not to the density of free DNA.

HPV-11 was purified in bulk from experimental grafts by the following preparative method. A second extract (CA 3) of the experimental condylomata was first produced. Keratin

TABLE 1. Infectivity of serial dilutions of natural and experimental condylomatous extracts with respect to human foreskins

Virus stock	No. infected/total no. of grafts at a virus dilutions of ^a :		
	0	10^{-1}	10^{-2}
CA 1 (natural)	6/8	2/6	4/10
CA 2 (experimental)	6/8	3/5	3/8

^a Number of foreskin grafts which were condylomatous per total number of foreskin grafts which were viable at the time of harvest (90 days).

FIG. 3. CsCl density gradient of HPV-11 suspension. CA 2 extract (500 μ l) was mixed with CsCl and centrifuged. No light-scattering bands were visible in the gradient. The radioactivity plotted is the amount of HPV-11 32 P-labeled DNA probe bound under stringent conditions. Maximal DNA binding was found at a density of 1.34 g/ml (g/cc).

cores from 21 foreskin grafts which had been infected with HPV-11 about 150 days previously were pooled, weighed (total of 15.3 g), and ground with sterile sea sand and PBS with a mortar and pestle. The resultant paste was diluted to 72 ml with PBS and subjected to three cycles of low-speed (10,000 rpm) and high-speed (40,000 rpm) centrifugation in an ultracentrifuge equipped with a SW41TI rotor (Beckman Instruments, Inc., Fullerton, Calif.) (1). Pellets from low-speed spins were discarded, and the supernatants were

sedimented to pellet virions. High-speed pellets were dispersed by pipetting and 10 min of sonication at 0°C and were diluted up to the original volume of PBS. The final high-speed pellet was suspended in 4.5 ml of NT buffer (0.05 M Tris base, 0.13 M NaCl [pH 7.8]); 2.0 g of CsCl was added, and the solution was mixed thoroughly and centrifuged overnight in an SW50.1 rotor at 45,000 rpm. Examination of the CsCl gradient with a perpendicular light beam demonstrated an opalescent band about 1 mm thick, located at a density of 1.34 g/ml (Fig. 4A). Fractions of 0.25 ml were collected by bottom puncture of the gradient, and 50 μ l of each fraction was spotted on cellulose nitrate paper. The fractions were subjected to alkali to denature the protein and render the DNA single stranded (3). The samples were hybridized with a 32 P nick-translated HPV-11 probe as previously described (7). The results were identical to the data presented in Fig. 3, except that the amount of virus DNA was much greater. This reflects the difference in the original inoculum. Fraction 5 was then dialyzed overnight against NT buffer. A drop was placed on Formvar-coated electron microscopy grids, along with 2% phosphotungstic acid in distilled water (pH 7.2). Virions were abundant in the preparation (Fig. 4B), and their appearance was consistent with that of an HPV (10). There were insufficient purified virions for an assay of infectivity.

This report demonstrates the first laboratory production of an HPV. Experimentally induced condylomata were microscopically identical to those which developed naturally in patients. Koilocytotic nuclei of experimental lesions contained substantial amounts of capsid antigen and virions. Experimental condylomata had HPV-11 DNA concentrations and infectivity very similar to those of the natural lesions. The extract of experimental condylomata was highly successful in three experiments in transmitting the infection to a new generation of human foreskin grafts. This observation indicates that it is now possible to produce HPV-11 in the laboratory. That experimental condylomata extracts were infectious at a dilution of 10^{-2} indicates that a substantial amount of HPV was produced. In the Shope papil-

FIG. 4. Purification of HPV-11 virions in CsCl density gradients. (A) Photograph of opalescent band located in CsCl density gradient at 1.34 g/ml. (B) Electron micrograph of phosphotungstic acid-impregnated preparation of virions obtained from the opalescent band shown in panel A.

lomavirus system, an infectious dose for 50% of rabbits corresponds to about 10^8 viral particles (2). It seems likely that a similar figure prevails in the HPV-11 system. The development of the condylomata as cysts may be advantageous, since all of the keratin layers produced by the transformed cysts are retained within the lesions and are not externally desquamated, as with exophytic lesions in the orthotopic location. These keratinized squames are a rich source of complete virions, as we have demonstrated with immunoperoxidase reagents for capsid antigen and by electron microscopy. The demonstration of experimental condylomatous extract infectivity is significant for another reason, since it is the first formal fulfillment of the fourth postulate of Koch in support of the HPV-11 etiology of the condylomata (6) in the original patients.

Virions purified from PBS extracts of the experimental condylomata had the morphology, density, and DNA content of HPV-11. The ability to produce and purify HPV in the laboratory opens the way to the study of the proteins and antigens of the capsid. It has not been possible until now to obtain sufficient quantities of HPV virions other than HPV-1 to make studies feasible.

Since capsid proteins are encoded by the late regions of the HPV DNA, it should also be possible to investigate viral and host factors which regulate expression of those portions of the HPV genome. For example, we do not know that experimental production of infectious virions in this system is maximal. Most human warts contain very little HPV (4), so it is not clear how or why viral replication is suppressed in natural lesions. Future analysis will reveal optimal conditions, which could allow the maximal laboratory production of HPV-11 and possibly other HPV.

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