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Rapid Communication

Human papillomavirus type 45 propagation, infection, and neutralization

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Abstract

The organotypic (raft) culture system has allowed the study of the entire differentiation-dependent life cycle of human papillomaviruses (HPVs), including virion morphogenesis. We introduced linearized HPV45 genomic DNA into primary keratinocytes, where it recircularized and maintained episomally at a range of 10–50 copies of HPV genomic DNA. Following epithelial stratification and differentiation in organotypic culture, virion morphogenesis occurred. HPV45 virions were purified from raft cultures and were able to infect keratinocytes in vitro. By testing a panel of HPV VLP antisera, we were able to demonstrate that the infection was neutralized not only with human HPV45 VLP-specific antiserum, but also with human HPV18 VLP-specific antiserum, demonstrating serological cross-reactivity between HPV18 and HPV45.
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Introduction

Cervical cancer is the most common cancer in developing countries and the second most common cancer in women worldwide (Mohar and Frias-Mendivil, 2000). Over 90% of all cervical cancers examined have been associated with human papillomaviruses (HPVs) (zur Hausen, 1989, 1991). Approximately one-third of HPVs infect the genital epithelium with a subset, including types 16, 18, 31, 33, 45, and 56, that is associated with an increased risk of cervical malignancy (Lorincz et al., 1992). Intermediate-risk HPV31 and 33 are more often associated with intraepithelial lesions than with invasive cancers, while high-risk HPV16, 18, 45, and 56 are more predominantly associated with invasive cancer (Lorincz et al., 1992). Two discrete patterns of risk, however, are found within this high-risk group. Compared to HPV16, HPV18, 45, and 56 are overrepresented in invasive tumors (Lorincz et al., 1992). In addition to being overrepresented, it is also known that infection with HPV18 confers a poor prognosis to the patient (Burger et al., 1996). HPV18 shares 25% homology with HPV45 based on reassociation kinetics (Naghashfar et al., 1987), a degree of homology that is similar to that which is found between HPV6 and HPV11 (Gissmann et al., 1982). While HPV45 is found in 8% of all cervical cancer cases, it is in significant excess in western Africa (Bosch et al., 1995). Thus, the entire life cycle of HPV45, which is the third most common high-risk HPV (Bosch et al., 1995), is of interest to study, as it appears to have important implications in cervical carcinogenesis, particularly in western Africa.

The complete life cycle of HPV is tightly linked to the differentiation program of the host epithelium (Grussendorf and zur Hausen, 1979; Howley, 1996; Meyers et al., 1992, 1997). The organotypic (raft) epithelial culture system has been used to develop an in vitro system capable of reproducing the complete viral life cycle (Meyers et al., 1992, 1997). This system has been used to study viral promoter activity, viral mRNA expression,

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viral mRNA splicing patterns, viral DNA amplification, late gene expression, and virion morphogenesis during the complete life cycle of HPV31b (Frattini et al., 1996; Meyers et al., 1992; Ozbun and Meyers, 1997, 1998a, 1998b, 1999a, 1999b). Additionally, infectious stocks of HPV31b, HPV18, and chimeric HPV18/16 have also been produced using this system, demonstrating the ability of this system to produce stocks of HPV for infectivity and neutralization studies (Meyers et al., 1992, 1997, 2002). The ability to reproduce the entire HPV life cycle, and ultimately produce infectious virus, will allow researchers to further understand the differentiation-dependent life cycle of HPV. In addition, these studies can provide insight into the early stages of infection, providing important information useful for developing therapeutics and vaccines. Virus produced using this system can be used in the place of artificial pseudovirion constructs in neutralizing assays, allowing for a more realistic representation of the in vivo situation. We report the use of this system to produce HPV45 cell lines capable of completing the complete viral life cycle, including virion morphogenesis, and the production of infectious HPV45, which could be neutralized by both human HPV18 and HPV45 VLP antisera.

Results

Development of cell lines maintaining episomal copies of HPV45 genomic DNA

Cell lines maintaining episomal copies of HPV45 were created utilizing an electroporation technique that has been developed and previously used by our laboratory to establish cell lines maintaining episomal copies of wild-type HPV18 and chimeric HPV18/16 (Meyers et al., 1997, 2002). The pHPV45 plasmid was digested with *Hin*dIII which both linearized and separated the HPV45 genomic DNA from vector DNA. Multiple batches of pooled primary human foreskin keratinocytes (HFK) were electroporated with the linearized HPV45 DNA. Individual electroporations were selected by immortalization and allowed to expand in culture. Recircularization and subsequent maintenance of episomal HPV45 viral genomes were confirmed by Southern blot (Fig. 1). Cell lines maintained the HPV45 genomic DNA episomally at approximately 10–50 copies/cell (Fig. 1).

Total DNA was isolated from the cell lines after several weeks of growth in culture. A minimum of 20 population doublings occurred during this growth period, corroborating the fact that stable vDNA replication and episomal maintenance were occurring. While we cannot rule out the possibility that integrated copies of HPV45 exist in these cell lines, they were undetectable and our data indicate that the HPV45 DNA is predominantly episomal. This result once again validates the ability of our system to produce cell lines

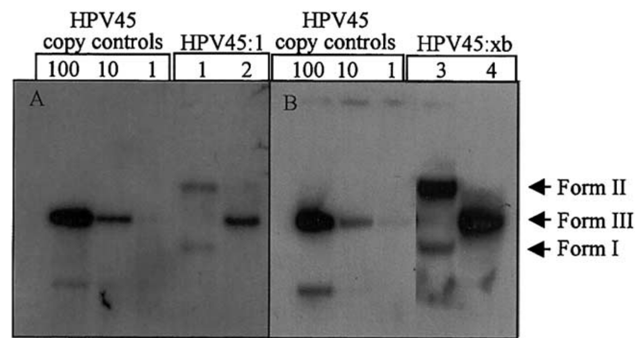

Fig. 1. Southern (DNA) blot hybridization of HPV45 DNA electroporated HFK cell lines grown in monolayer culture. Five micrograms of total cellular DNA from HPV45 electroporated HFK cell lines HPV45:1 and HPV45:xb was separated by electrophoresis on an 0.8% agarose gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled HPV45 genomic probe. Samples in lanes 1 and 3 were digested with *Eco*RI, a noncutter of the HPV45 genome. Samples in lanes 2 and 4 were digested with *Eco*RV, a single cutter of the HPV45 genome. HPV45 100, 10, and 1 copy number standards are shown. Arrows indicate Form II DNA (nicked), Form III DNA (linear), and Form I DNA (supercoiled).

with episomal replicating HPV DNAs (Meyers et al., 1997, 2002). Approximately 50% of the electroporations yielded cell lines in which the HPV45 genome was maintained episomally, while the other 50% of the lines did not contain detectable HPV45 DNA. While this is similar to other HPVs studied in this system (unpublished data), it is in contrast to HPV18 and HPV18/16 chimeras, in which 100% of the cell lines electroporated maintained episomes (Meyers et al., 1997).

We next attempted to subclone the HPV45:xb line, as this line contains a heterogeneous population of cells. Despite the fact that we have successfully subcloned HPV18, HPV18/16, HPV33, and HPV39 containing cell lines (Meyers et al., 1997, 2002), the HPV45:xb subclones failed to grow in culture. While the underlying reason for this failure to subclone remains unknown, we have shown in other assays that HPV45 does not behave like other HPVs (unpublished data).

Morphology of HPV45 cell lines grown in raft cultures

A representative HPV45 cell line, HPV45:xb, was chosen for further study. We were interested in examining the effect of HPV45 on tissue differentiation and morphology, as the life cycle of HPV is tightly linked to the differentiation program of the host tissue. HPV45:xb was allowed to stratify and differentiate in the raft culture system, and tissue sections were stained with hematoxylin and eosin to observe tissue morphology. Occasional koilocytes and periodic parakeratotic bodies and a normal looking cornified layer could be observed (Fig. 2). The raft tissue was next stained with the differentiation marker involucrin. The presence of involucrin indicated that the differentiation program of the tissue had indeed begun (Fig. 2).

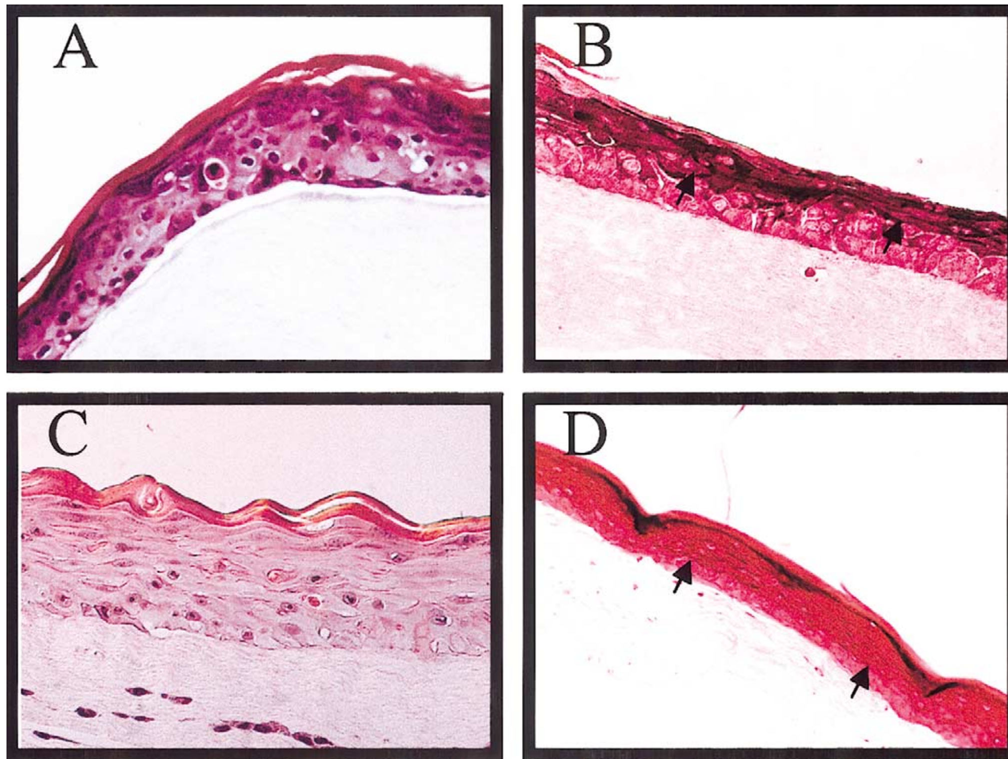

Fig. 2. Immunohistochemical analysis of HPV45:xb raft culture tissue. HPV45:xb fully stratified and differentiated tissue was stained with hematoxylin and eosin (A) or immunostained with involucrin polyclonal antiserum (B). HFK fully stratified and differentiated tissue was stained with hematoxylin and eosin (C) or immunostained with involucrin polyclonal antiserum as a control (D).

Infectious HPV45 biosynthesis

Virion morphogenesis represents the final stage of the viral life cycle. HPV45:xb raft culture tissues were grown and putative viral stocks were prepared to investigate the ability of this line to produce infectious virus particles. HaCaT cells were infected with dilutions of HPV45 stocks. The cells were harvested after 48 h of growth and virus infection was detected by the presence of the HPV45 spliced E1^E4 transcript represented by a nested PCR product of approximately 400 bp (Fig. 3). HPV45 was infectious to a 1:2000 dilution (Fig. 3).

Neutralization analyses

After determining that our HPV45 stock was infectious, we wanted to determine whether antisera raised against a variety of HPV VLPs were capable of neutralizing HPV45 viral infection. HPV45 was incubated with a panel of antisera raised in rabbits against HPV6, 11, 16, 18, 31, 33, 39, and 45 VLPs (Fig. 4). The indicated VLP antiserum was diluted 1:100 and preincubated with diluted HPV45. The antisera–virus mix was then incubated with HaCaT cells for 2 days at which time RNA was extracted and virus infection

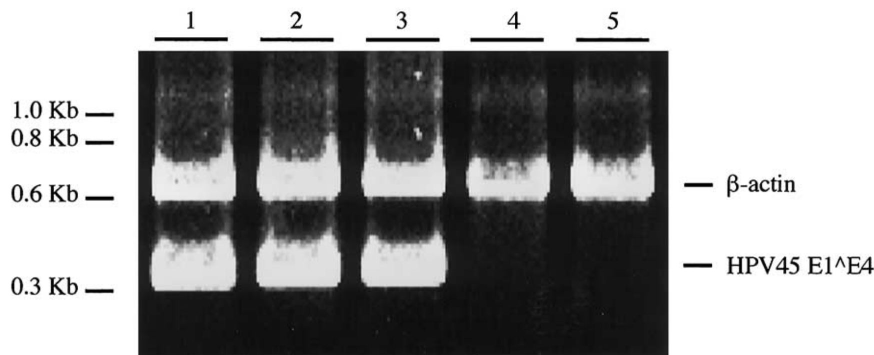

Fig. 3. Infectious titer of HPV45:xb. Shown is a 2% agarose gel of nested RT-PCR-amplified HPV45 E1^E4 and β-actin. Lanes 1 to 4, HPV45:xb at 1:500, 1:1000, 1:2000, and 1:4000, respectively. Lane 5, negative control (no virus). β-Actin and E1^E4 are indicated on the right and molecular size markers are indicated on the left.

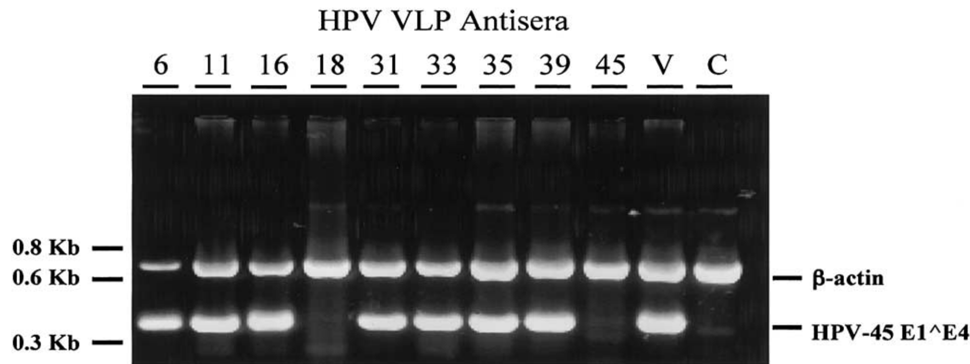

Fig. 4. Neutralization of HPV45:xb. Shown is a 2% agarose gel of nested RT-PCR-amplified HPV45 E1^E4 and β-actin. β-Actin and E1^E4 are indicated on the right and molecular size markers are indicated on the left. HPV45 was preincubated with the indicated HPV VLP antisera or with normal rabbit serum (V). C indicates uninfected cells.

was again detected by the presence of an approximately 400-bp HPV45 E1^E4 nested PCR product. HPV45 was neutralized by antisera raised against HPV45 VLPs and HPV18 VLPs as demonstrated by the inability to detect the E1^E4 spliced message. Antisera raised against VLPs composed of L1 from HPV6, 11, 16, 31, 33, 35, and 39 failed to neutralize HPV45 (Fig. 4).

We next wanted to determine the specific HPV45 neutralizing activity of the antisera raised against HPV18 and HPV45 VLPs. HPV45 was preincubated with serial $\log_{10}$ dilutions of anti-HPV18 VLP sera or with serial $\log_{10}$ dilutions of anti-HPV45 VLP sera. Antisera against HPV18 VLPs could be diluted to 10^{-4} and still neutralize HPV45 infection and antisera raised against HPV45 VLPs could be diluted to 10^{-5} and still neutralize HPV45 infection (Fig. 5). As the antisera are uncharacterized, however, the specific activities cannot be directly compared to one another but suggest that the HPV45 neutralizing abilities of both antisera are similar.

Discussion

Until the establishment of the organotypic (raft) culture system for the propagation of HPV31b (Meyers et al., 1992), the ability to effectively produce abundant infectious HPV stocks and thus to study the complete viral life cycle was limited. Taken a step further, the raft culture system was coupled with DNA electroporation and shown to yield infectious HPV18 and HPV18/16, conferring the ability to propagate preselected HPV types in vitro (Meyers et al., 1997, 2002). We have now described the use of the electroporation-based system to create cell lines capable of completing the entire life cycle of HPV45. When the HPV45 DNA was introduced into primary keratinocytes it was maintained episomally at a range of 10 to 50 copies per cell. Southern blot analysis confirmed that the vDNA was stable. When a representative HPV45 line was allowed to stratify and differentiate in our raft culture system, virion morphogenesis occurred.

Stocks of HPV45 virions were prepared that could infect human keratinocytes in vitro. The infectivity of the HPV45 stocks was neutralized not only with antibodies raised against HPV45 VLPs, but also with antibodies raised against HPV18 VLPs. These data demonstrate antigenic relatedness between HPV18 and HPV45. This finding is not surprising, as it has been established that HPV18 and HPV45 are closely related phylogenetically. In fact, HPV18 shares 25% sequence homology with HPV45 based on reassociation kinetics (Naghashfar et al., 1987), a degree of

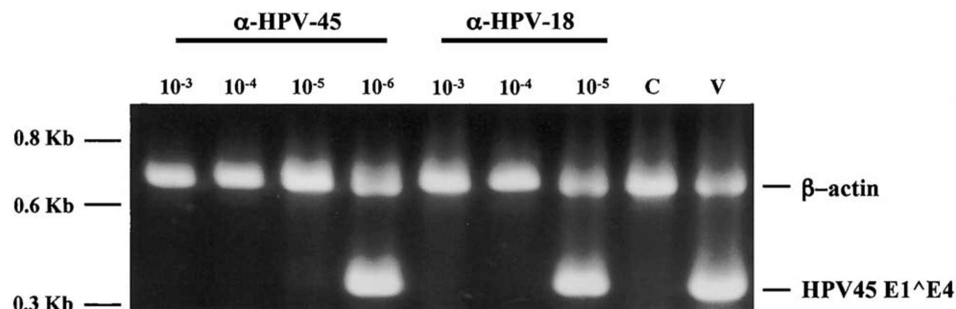

Fig. 5. Titration of the HPV45 neutralizing activity in the anti-HPV45 and anti-HPV18 VLP sera. Shown is a 2% agarose gel of nested RT-PCR-amplified HPV45 E1^E4 and β-actin. β-Actin and E1^E4 are indicated on the right and molecular size markers are indicated on the left. HPV45 anti-sera was diluted 10^3, 10^4, 10^5, and 10^6 and the HPV18 anti-sera was diluted 10^3, 10^4, and 10^5. C, no virus; V, normal rabbit serum.

homology that is similar to that which is found between HPV6 and HPV11 (Gissmann et al., 1982). These two closely related viruses have also been shown to be overrepresented in invasive tumors (Lorincz et al., 1992). Additionally, it has been shown that HPV18 is associated with poor patient prognosis (Burger et al., 1996). An interesting question that remains to be addressed is whether infection with HPV45 also confers a poor prognosis to the patient. Further study into the similarities and differences between the virions and viral life cycles of HPV18 and HPV45 would be highly informative. Since HPV18 has previously been produced in culture and with the ability to synthesize HPV45 virions, we are able to study these similarities and differences more closely. We have now shown with neutralization studies that in fact there is highly evident cross-reactivity of surface antigens from both HPV18 and HPV45 virions, demonstrating similarities in immunological response. This cross-reactivity may in fact prove beneficial when developing vaccine strategies against these related viruses, as a prophylactic vaccine against HPV18 may protect against HPV45 and vice versa.

In combination with previous studies, we have now established a system to study and compare the life cycles of HPV18 and HPV45. We are also now in the position to perform genetic analyses of HPV18 and HPV45 in parallel in order to determine which features of the viral life cycles are similar and different between these two viruses. Additionally, our infectious stocks of HPV18 and HPV45 now allow us to investigate differences in the early stages of infection of these two viruses. Information gained from these studies will be useful not only for the understanding of the viral life cycles of different HPVs, but also for the development of antiviral therapies.

Materials and methods

Keratinocyte and organotypic raft cultures

Primary HFK were isolated from newborn circumcision. The dermis was removed from the epidermis and discarded. The epidermis was washed three times with PBS containing 50 μg/ml gentamycin sulfate (Gibco BRL, Bethesda, MD) and 1$\times$ nystatin (Sigma Chemical Co., St. Louis, MO). The epidermis was then minced and trypsinized into a single cell suspension in a spinner flask. Keratinocytes were grown in 154 medium (Cascade Biologics, Inc., Portland, OR) supplemented with Human Keratinocyte Growth Supplement kit (Cascade Biologics, Inc.). Keratinocyte lines stably maintaining HPV45 DNA following electroporation were grown in monolayer culture using E medium in the presence of mitomycin C treated J2 3T3 feeder cells (Meyers, 1996; Meyers et al., 1992, 1997).

Organotypic (raft) cultures were grown as previously described (Meyers, 1996; Meyers et al., 1992, 1997). Briefly, cell lines were seeded onto rat tail type 1 collagen matrices containing J2 3T3 feeder cells. Following cell attachment and growth to confluence, the matrices were lifted onto stainless-steel grids. Once at the air–liquid interface the raft cultures were fed by diffusion from below with E medium supplemented with 10 μM 1,2-dioctanoyl-*sn*-glycerol (C8:O; Sigma Chemical Co.). Raft cultures were allowed to stratify and differentiate for 12 days, as viral gene expression has been shown to peak between 10 and 12 days in the raft system (Ozbun and Meyers, 1997).

Electroporation of primary keratinocytes

Primary human foreskin keratinocytes were electroporated as previously described (Meyers et al., 1997, 2002). Briefly, pHPV45 plasmid DNA, a generous gift from A. Lorincz (Naghashfar et al., 1987), was digested with *Hin*dIII, linearizing the viral DNA and separating it from vector sequences. A total of 10 μl of the *Hin*dIII-digested DNA (1 μg/μl) was mixed with 4.25 μl of sonicated and denatured salmon sperm DNA (10 μg/μl) in a 1.5-ml Eppendorf tube. A total of 5×10^6 keratinocytes in a volume of 250 μl of E medium containing 10% fetal bovine serum (FBS) and 5 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid were added to the DNA mixture. The DNA and keratinocyte solution was transferred to an electroporation cuvette and electroporated using a Gene Pulser (Bio-Rad Laboratories, Hercules, CA) set at 210 V and 960 μF. The electroporated cell solution was then layered into 10 ml of E medium containing 10% FBS and centrifuged at 25*g* for 10 min. The medium was removed and the cell pellets were resuspended in E medium containing 10% FBS. The suspensions were then added to 10-cm tissue culture plates containing mitomycin-C-treated J2 3T3 feeder cells. A total of 5 ng of EGF/ml was added to the culture medium the next day. The cultures were fed every other day for 7 days with E medium containing 10% FBS and 5 ng EGF/ml. After the 7-day period, the cells were fed with E medium containing 5% FBS and 5 ng EGF/ml until the keratinocytes grew to confluence, at which time the EGF was omitted from the medium.

Southern (DNA) blot hybridization

Total cellular DNA was isolated as previously described (Meyers et al., 1992; Ozbun and Meyers, 1998a). A total of 5 μg of total cellular DNA was digested with either *Eco*RI, which does not digest the HPV45 genome, or *Eco*RV, which linearizes the HPV45 genome at nucleotide 2234. The samples were then separated by 0.8% agarose gel electrophoresis and transferred onto a GeneScreen Plus membrane (New England Nuclear Research Products, Boston, MA) as previously described (Ozbun and Meyers, 1998a). Hybridization of the Southern blot was performed as previously described (Meyers et al., 1992; Ozbun and Meyers, 1998a), probing with an HPV45-specific complete genomic probe.

Histochemical analyses

Raft cultures were grown for 12 days, harvested, fixed in 10% neutral buffered formalin, and embedded in paraffin. Four-micrometer sections were cut and stained with hematoxylin and eosin as previously described (Meyers et al., 1992). Immunostaining was done using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame) according to the manufacturer's instructions. Involucrin polyclonal antiserum was used for immunostaining (1:5; Biogenesis, Sandown, NH).

Virus isolation and in vitro infectivity assay

Virions were isolated as previously described (Meyers et al., 1997, 2002). The HPV45 infectivity studies were based on an in vitro system described by Smith et al. (Smith et al., 1995). HaCaT cells, an immortalized human keratinocyte cell line (kindly provided by Norbert Fusenig), were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin in 24-well plates. The HPV45 virus stock was sonicated for 30 s on ice and then serially diluted with cell culture medium. The medium was aspirated from the HaCaTs and 0.5 ml of each dilution of virus stock was added per well. One well on each plate received 0.5 ml of medium without virus as a control. The cells were incubated with the virus for 48 h at 37°C.

The ability of the HPV45 virus to infect the HaCaT cells after 48 h of incubation was determined by the presence of the spliced HPV45 E1^E4 mRNA species (Smith et al., 1995). mRNA was purified from the infected cells using the mRNA capture kit (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, the medium was aspirated from the cells and the cells were washed two times with 0.5 ml ice-cold 1× PBS. The final PBS wash was aspirated from the cells and 0.25 ml lysis buffer was added to each well. The cell lysates were removed from the wells and sonicated for 2 min in a cup horn sonicator on ice. A total of 1 μl of 1:4 diluted biotinylated oligo dT was added to each lysate. The samples were incubated for 10 min at 42°C. A total of 50 μl of the lysate was transferred to a streptavidin-coated PCR tube and incubated for 3 min at 37°C. The RNA captured in the tubes was washed three times with 200 μl of wash buffer and subsequently used in a RT reaction utilizing reagents from the First Strand cDNA kit (Roche Molecular Biochemicals). The cDNA was then used for nested PCR to detect the HPV45 E1^E4 cDNA. Forty cycles of PCR were performed on the cDNA using 5′GAGCTTA-CAGTAGAGAGCTCG3′ as the forward primer (located at nucleotide position 806–826 in the HPV45 genome) and 5′ TGTTACCACTACACACTTTCCTTC3′ (located at nucleotide 3613–3636 in the HPV45 genome) as the reverse primer. Ten percent of the first PCR mixture was used as template for 40 cycles of nested amplification utilizing 5′GCAGAGGACCTTAGAACACTA3′ (located at nucleotide position 827–847 in the HPV45 genome) as the forward nested primer and 5′GAACACAGGAGCGGGTT-GTGC 3′ (located at nucleotide position 3572–3592 in the HPV45 genome) as the reverse nested primer. An additional set of primers specific for β-actin was included in the PCR mixture as a control for mRNA detection. The forward primer for the first reaction was 5′GAACCCCAAGGC-CAACCGCGA3′ and the reverse primer was 5′CCACA-CAGAGTACTTGCGCTCAGG3′. The forward primer for the nested reaction was 5′GATGACCCAGATCAT-GTTTG3′ and the reverse primer was 5′GGAGCAT-GATCTTGATCTTC3′. All PCR reactions contained 10 mM Tris–HCl, pH 8.3, 50 mM KCL, 2.5 mM MgCl2, 200 μM dNTPs, 125 ng of each forward and reverse primer, and 2.5 units of Taq polymerase (PE Biosystems). The temperature profile for all reactions was 95°C for 2 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, with a final 10-min extension at 72°C. All PCR products were visualized by electrophoresis in a 2% agarose–ethidium bromide gel. The virus titer was determined to be the last dilution at which the spliced transcript could be detected.

Neutralization of HPV45

The neutralization experiments were performed by diluting the HPV45 stock and preincubating the diluted virus for 1 h with a panel of 1:100 diluted anti-VLP sera produced in rabbits as previously described (White et al., 1998). The panel included HPV6, 11, 16, 18, 31, 33, 39, and 45 L1 VLP antisera. Following the preincubation, HaCaT cells were infected with the virus–antibody mixture and incubated for 2 days, at which time RNA was extracted and the E1^E4 transcript was detected as described above.

Acknowledgments

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