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Biochemistry

A rhesus monkey model for sexual transmission of a papillomavirus isolated from a squamous cell carcinoma

(molecular and pathological analyses/retrospective study/malignant tumor)

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ABSTRACT **Recently we molecularly cloned and characterized a papillomavirus from a lymph node metastasis of a primary penile carcinoma found in a rhesus monkey; this virus species, rhesus papillomavirus type 1 (RhPV-1), is similar to oncogenic human papillomaviruses (HPVs), such as HPV-16 or HPV-18, in that the RhPV-1 DNA was found to be integrated in the tumor cell DNA. To compare the sexual transmission and oncogenic nature of RhPV-1 with these HPVs, we undertook an extensive retrospective study of a group of rhesus monkeys whose sexual mating and offspring histories were known. These animals had mated directly with the index male mentioned above or were secondarily exposed to this virus through intermediate sexual partners. This study combines cytological, histopathological, and several complementary hybridization and DNA amplification techniques on multiple tissue samples to demonstrate the sexually transmitted nature of RhPV-1. The oncogenic potential of RhPV-1 is suggested in several of the infected animals by the presence of various degrees of neoplasia including squamous cell cancer of the cervix.**

The compelling evidence that certain types of human papillomavirus (HPV) are associated with malignancies of the anogenital tract has been based largely upon the detection of HPV DNA in various tissues (for review, see ref. 1). *In vitro* molecular studies have corroborated these results (1), demonstrating that DNA from certain HPV types can transform cells, thereby providing experimental evidence that these viruses impart neoplastic qualities to infected tissues. Papillomaviruses are necessary, but not alone sufficient, for malignant transformation. The predominant types associated with malignant lesions include HPV-16, -18, and -31 (2–4) and, to a lesser degree, HPV-33 and -35 (5, 6) and are usually found integrated into the host genome. In contrast, HPV-6 and HPV-11 DNAs have been observed as free episomes in both benign genital and laryngeal papillomas (7, 8); yet both DNAs in altered episomal or integrated forms have been associated with malignant diseases (9–12). All of these genital papillomaviruses are widespread in the general population. By various DNA filter hybridization techniques, surveys of clinically normal women estimate that 5–50% can be found to harbor papillomaviruses in their genital tracts (13–17). The sexually transmitted nature of this clinically significant HPV-associated disease is apparent from the fact that up to 65% of male consorts of women with genital warts or neoplasia also have warty or neoplastic lesions (18, 19).

Although there are animal model systems to study warty cutaneous diseases, such as cottontail rabbit papillomavirus, there is no current animal model appropriate for genital oncogenic papillomaviruses. We recently isolated and characterized a species of papillomavirus from a rhesus monkey that appears to serve as a useful model for sexual transmission and oncogenicity of papillomavirus infection of primate genitalia (20). We undertook a series of complementary studies that probed the extent and biological significance of naturally occurring RhPV-1 infections in a population of female rhesus monkeys sexually active with an index male infected with RhPV-1 that had developed malignant tumors (20).

MATERIALS AND METHODS

DNA Extraction from Tissue and Filter Hybridization Analysis. Frozen tissue samples were extracted for total cellular DNA as described (12) with slight modifications. Tissue was incubated overnight at 37°C with SDS and protease and either treated with ribonuclease, extracted with phenol and chloroform and ethanol precipitated or pooled cellular and episomal viral DNA was isolated by isopycnic banding in CsCl gradients containing ethidium bromide, butanol extraction, and isopropanol precipitation. After transfer to nitrocellulose filter, stringent hybridization using a ^{32}P-labeled RhPV-1 DNA probe was done, as described (Fig. 1) (12). Controls included 20 pg of RhPV-1 plasmid DNA cleaved with *Bam*HI and ≈10 μg of negative control human placenta DNA.

***In Situ* Hybridization.** *In situ* hybridization was done as described (21).

Polymerase Chain Reaction (PCR) Amplification. A GeneAmp kit (Perkin–Elmer/Cetus) was used following the manufacturer's instructions with two oligonucleotide primers derived from the L2 region of RhPV-1 (R.S.O., unpublished data) by using 32 cycles of amplification. These primers are a 15-mer, RO1A (5′-TGGCATTGCACAGGC-3′), and an 18-mer, RO3 (5′-CCACCTTAGACACAGGAC-3′). A portion of the amplified product was then analyzed by Southern blot hybridization with a ^{32}P-labeled RhPV-1 subgenomic DNA probe specific for the amplified region (Fig. 1). Based upon our recent sequencing of the complete RhPV-1 genome (R.S.O., unpublished data), another set of primers derived from the upstream regulatory region (5′-CAGCTTGCCTGCTGCAC-3′) and E6 region (5′-CGCCAGGGCAGTCTACC-3′) of RhPV-1 were used on those samples positive by Southern blotting but negative by PCR analysis with the first set of primers; several positive and negative controls were run as well as repeat analyses of some other samples.

RESULTS

This study retrospectively examined several groups of rhesus monkeys for evidence of papillomavirus infection. These

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Abbreviations: RhPV-1, rhesus papillomavirus type 1; HPV, human papillomavirus; PCR, polymerase chain reaction.

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consisted of 30 females and 1 sexually active male in the mating group of the index male 843 (20) (Fig. 2). The index male, subsequent to these mating activities, developed a penile carcinoma that metastasized to the lymph node. Control animals consisted of four mature females from a distinct sexually active cohort and 7 virgin mature female rhesus monkeys. Thus, the total number of animals in this study was 41 females and 2 males, including the index male.

Initial Detection of RhPV-1 DNA in Sexual Partners of Index Male Rhesus Monkey. Our initial studies were conducted on a subset of 22 of the 41 female rhesus monkeys. Eleven animals had been mated directly to the index male; the others were animals that either mated outside of this group or not at all. All analyses were performed and scored blind to the mating histories. Exfoliated cervical epithelium was collected for cytologic examination and DNA extraction. By using a molecularly cloned RhPV-1 DNA probe, stringent Southern blot hybridization was done with DNA digested by *Bam*HI, a restriction endonuclease with a single cleavage site within the RhPV-1 genome. Samples from two females sexually active with the index male demonstrated the presence of unit-length, 8-kilobase (kb) genomes. One of the samples contained several other smaller bands at the single-copy level that may be due to subgenomic episomal DNA or integration into the cellular genome (data not shown).

In Depth Cytological, Pathological, and Molecular Analyses for Evidence of RhPV-1 Infection. More extensive sampling was then done on a subset of 32 animals, including other members of the mating cohort and several from our preliminary study group. Not all animals from the preliminary screening were available for repeat screening. These animals had either direct sexual contact with the index male or indirect contact through intermediate sexual partners, some of which were not part of this study. To ensure optimal detection of infection and disease and to reduce false negatives from sampling errors or insensitivity of any one detec-

Table 1. Clinical, histopathological, and molecular evidence for RhPV-1 infection in rhesus monkey

Animal	Mated*	Cytological class	Histopathology† (acetowhitening)	Filter hybridization		*In situ* hybridization	PCR results‡	
				Smear	Biopsy		Smear	Biopsy
735	In	1	ND	−	ND	ND	−	ND
746	In	2	Dysplasia, CIN 1	+	−	−	+	−
816	In	1	−	−	−	−	+	−
929	In	2	− (A)§	+	+	−	+	+
1126	In	1	ND	−	ND	ND	−	ND
1416	In	1	Koilocytosis	−	−	−	−	+
1422	In	1	−	+/−	−	−	+	+
AE32	In	1	−	+	+	+	+	+
AG19	In	2	−	+/−	−	−	+	+
AG26	In	1	−	+/−	−	−	+	−
AI09	In	1	Koilocytosis	−	−	−	+	−
H97	In	1	−	+	−	−	+	+
L88	In	1	ND	−	ND	ND	−	ND
PP30	In	1	−	−	−	ND	−	−
PP37	In	1	−	−	−	−	−	−
PP46	In	1	−	−	−	ND	−	−
PP91	In	1	−	−	−	+	+/−;+	−
PP93	In	1	Koilocytosis	−	−	−	+	−
PP98	In	2	Inv. Cancer	+/−	+	+/−	+	−
PP99	In	1	−	−	−	−	−	−
PP100	In	2	Dysplasia, CIN 1	+	−	+/−	+/−;+	−
PP106	In	2	Dysplasia, CIN 1	−	+/−	+/−	−	+
PP108	In	1	−	−	−	−	−	−
PP110	In	1	−	+	−	+	−;+	+/−
PP111	In	2	Dysplasia, CIN 1	−	+/−	+	−	−
PP112	In	1	Koilocytosis, CIN 1	−	+/−	−	+	−
PP113	In	1	−	+	−	+	+	−
S74 (male)	In	ND	ND	+	ND	ND	+	ND
U16	In	1	−	−	−	−	−	−
X04	In	1	Koilocytosis (A)	+	−	−	+	+
X06	In	1	− (A)	+	−	−	+	+
439	Out	1	ND	−	ND	ND	−	ND
PP10	Out	1	ND	−	ND	ND	−	ND
PP21	Out	1	−	−	−	ND	−	−
PP27	Out	1	ND	−	ND	ND	−	ND
AC39	N	1	ND	−	ND	ND	−	ND
AD86	N	1	−	−	−	ND	−	−
AG22	N	1	ND	−	ND	ND	−	ND
AG48	N	1	ND	−	ND	ND	−	ND
AG57	N	1	ND	−	ND	ND	−	ND
V34	N	1	−	−	−	ND	−	−
Z82	N	1	−	−	−	ND	−	−

ND, not determined; Inv, invasive; CIN 1, cervical intraepithelial carcinoma grade 1.
*In, mated within the sample cohort; Out, mated outside of the sample cohort; N, never mated.
†(A), acetowhite observed on cervix.
‡−; + or +/−;+, PCR was negative or marginal, respectively, for late gene primers and positive for early gene primers.
§Pathological sectioning of the anogenital tract of this animal revealed an invasive adenosquamous carcinoma of the endocervix; see text.

tion technique: (*i*) a cervical smear sample was obtained from the ectocervix and endocervix of each female, employing a cytobrush (22). These cells were divided for cytology preparations (Table 1) and extracted for total cellular DNA. A cytobrush was also used to collect cells from the distal urethra of male rhesus monkey S74 in this mating group. DNA samples were analyzed by filter hybridization as above (Fig. 1*A*, Table 1). (*ii*) The cervices were painted with 3% acetic acid, taking note of any signs of a glistening white stain characteristic of papillomavirus-infected tissue. Two separate biopsies were taken from these acetowhite patches; one 3-mm biopsy was used entirely for DNA extraction and analysis (Table 1), and the second adjacent biopsy was used for both histopathological analysis and *in situ* DNA hybridization (23) (Table 1). (*iii*) For the most sensitive detection technique, 0.5 μg of each sample and control DNAs was amplified by the PCR using two oligonucleotide primers derived from the L2 open reading frame region of RhPV-1 (Fig. 1*B*, Table 1). Appropriate early gene sequence information for the RhPV-1 genome was not known at the time of these experiments but later became available (see below). The mating relationships of the animals used in this study and their status with respect to RhPV-1 DNA and histopathological results is shown in Fig. 2.

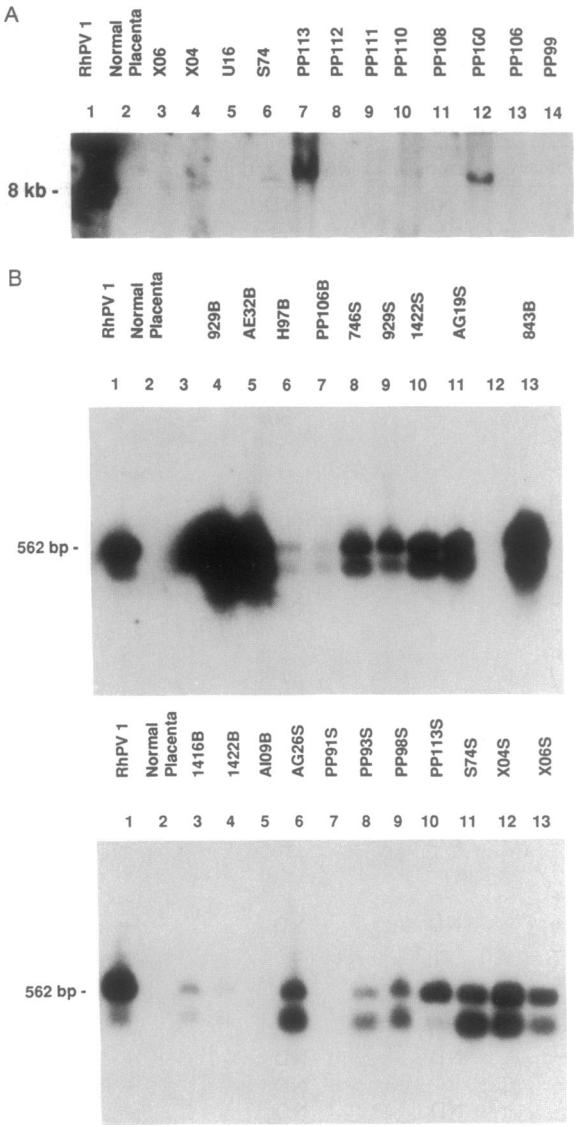

FIG. 1. Hybridization analysis of cervical samples. (*A*) Results of hybridization of DNA extracted from cells derived from cervical smears and cleaved with *Bam*HI. (*B*) Hybridization of PCR-amplified DNA product (562 base pairs in size) from cervical smears and biopsies. The faster migrating bands are an incomplete PCR product consisting of only one DNA strand. Either 4% or 20% of the entire PCR reaction product was used, so as to normalize adjacent samples and prevent cross-contamination. RhPV-1 indicates a positive control lane; normal placenta is a negative control. S or B after an animal's identification number refers to origin of the sample for PCR analysis (cervical scrape or biopsy, respectively).

Histopathologic Evidence for RhPV-1 Infection. Features of low-grade epithelial dysplasia in cytologic preparations observed in seven of these animals were taken as evidence of papillomavirus infection (Fig. 3 *A* and *B*; Table 1). All animals were either grade 1, which is normal, or grade 2, exhibiting an increased proportion of basal and parabasal cells, the presence of koilocytes, and occasional cells showing enlarged atypical nuclei. Histologic sections taken of cervical biopsies also revealed characteristic features of papillomavirus-associated neoplasia including koilocytosis, loss of maturation, nuclear atypia, and increased mitotic activity in 10 animals, one of which was found to have focally invasive, well-differentiated squamous cell carcinoma (Fig. 3*C*). One of these animals, 929, was sacrificed due to infirmity of old age, and a complete pathologic examination of the anogenital tract was performed. A large adenosquamous carcinoma of the endocervix was detected (Fig. 3*D*). In this case, the initial pathologic analysis underestimated the severity of disease. Only five animals were abnormal by analysis of both cytological preparations and biopsies. Three additional animals were also observed to have acetowhite lesions upon direct examination: one with koilocytosis upon biopsy, one with an abnormal smear (later found to have a cancer), and one with a normal biopsy and smear but that was positive for RhPV-1 DNA (Table 1). All 13 animals with positive clinical or pathological signs were also found to contain RhPV-1 DNA by one or more hybridization techniques (Table 1).

Detection of RhPV-1 DNA by Southern Blot Analysis of DNA Extracted from Tissues. Southern blot hybridization analysis of DNA extracted from either exfoliated cervical cells or biopsies showed positive evidence for the presence of RhPV-1 DNA, generally at very low-copy number (Table 1). In some cases the detection of viral DNA was only marginal and at the limits of detection for this technique [≈0.05 copy per cell (24)]. The squamous cell carcinoma of the cervix of animal PP98 revealed faint bands of high molecular weight which may be indicative of viral DNA integration into cellular DNA (data not shown).

Detection of RhPV-1 DNA by *in Situ* Hybridization of Biopsies. *In situ* DNA hybridization offers the advantage of identifying individual RhPV-1 DNA-positive cells containing a high-copy number of viral genomes within a largely RhPV-1 DNA-negative or low-copy number tissue (21). For tissues consisting of uniformly low-copy number cells, this technique is less sensitive than Southern analyses. By using ^{3}H-labeled RhPV-1 DNA, several biopsies were found to have positive cells by this technique (Table 1). The RhPV-1 DNA-positive cells, characterized as having grains of silver over nuclei, were few in number, and the detection was low-to-moderate in signal strength (data not shown), reflecting the overall low-copy number of RhPV-1 DNA in these biopsy specimens. In one case, *in situ* hybridization detected positive cells in animal PP91, where there was no pathological evidence for RhPV-1 infection, and was confirmed only by PCR amplification (see below).

Detection of RhPV-1 DNA After Amplification by PCR. To test for minute amounts of RhPV-1 DNA in the various samples, we used a PCR analysis coupled with DNA filter hybridization which, in control experiments, was easily able to detect ≈1 fg of RhPV-1 plasmid DNA. To avoid cross-contamination of samples, PCR analyses were performed by using positive-displacement pipettors and included inter-

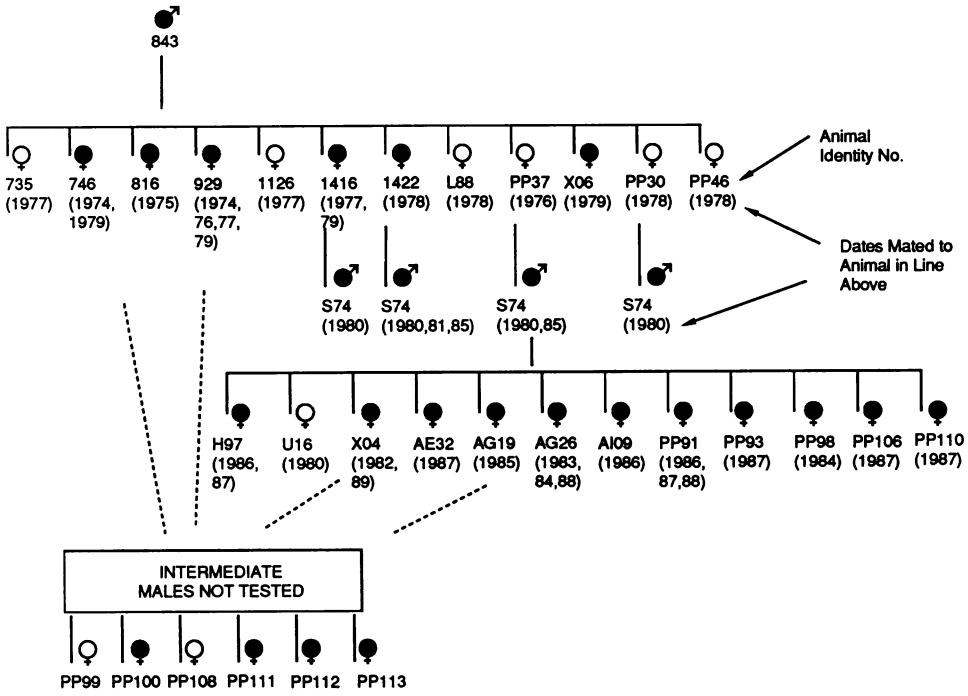

FIG. 2. Mating cohort relationships and clinical, histopathological, or molecular evidence for RhPV-1 infection. Shown are the most direct sexual relationships of the monkeys in this study and the date of the interaction. Positive animals (●) are those for which at least one clinical, histopathological, or molecular result indicated papillomavirus infection. ♀, Female; ♂, male, and ○, negative.

spersed samples of several negative control DNAs (human placenta and salmon DNA) and reagent controls. Multiple testing and rearrangement of samples was performed to ensure lack of contamination or adjacent lane spill-over. In most samples (80%) in which positive or marginal results were obtained by filter hybridization of the smear or biopsy DNA, PCR analysis was also positive. In five samples, this was not the case. However, two of these were only marginally positive (animals PP111, PP112), and four were samples derived from animals with dysplasia or invasive cancer (animals PP98, PP100, PP111, and PP112). As indicated above, the filter hybridization of cancer biopsy DNA (animal PP98) after *Bam*HI treatment revealed only high-molecular-weight DNA indicative of totally integrated RhPV-1 genomes and a lack of episomal viral DNA. PP110 had two bands that migrated slightly above and well below 8 kb, which may also indicate that an integration event had occurred (Fig. 1*A*). The resulting integration or other viral genomic alteration(s), often observed in dysplasias and cancers associated with oncogenic HPV DNAs, may have lost the target sequences necessary for the PCR priming. Usually integration of oncogenic papillomaviruses occurs in the E1 or E2 open reading frames with the subsequent loss of portions of the viral genome. At least one of the integration sites for RhPV-1 in a lymph node metastasis occurs within the L2 open reading frame. The nucleotide sequences of the oligonucleotide primers used in the current study were shown to exist within the integrated viral DNA, which has now been characterized

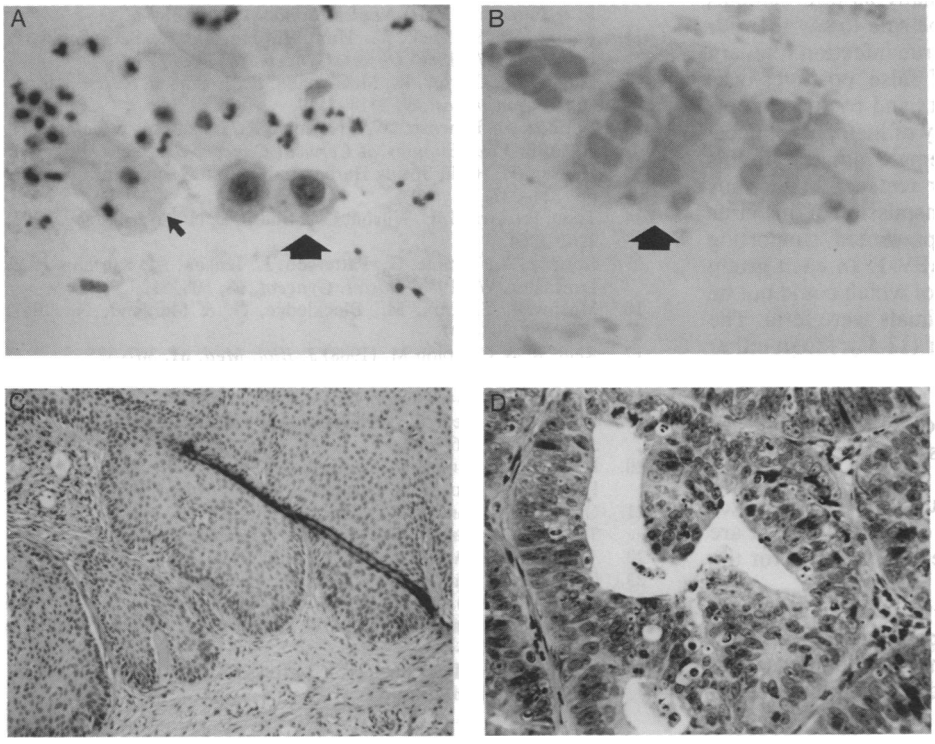

FIG. 3. Histopathological analysis of cervical smears and biopsies. Typical class 2 smears showing abnormal staining and large nuclear-to-cytoplasmic ratios (heavy arrow) are shown from animals AG19 (*A*) and PP98 (*B*) (×160 Papanicolaou). Small arrow indicates a normal cell in the same smear. PP98 was found to have an invasive squamous cell carcinoma of the cervix upon biopsy (*C*) (×40 hematoxylin/eosin). This tissue was characterized by neoplastic transformation of the full thickness of the cervical squamous epithelium comprised of focal undifferentiated cells with a pushing border and islands of invasion of the underlying fibrovascular stroma. Complete pathological analysis of the anogenital tract of animal 929 revealed a poorly to moderately differentiated adenosquamous carcinoma of the endocervix (*D*) (×160 hematoxylin/eosin), characterized by atypical and complex glands that lie in a back-to-back conformation and were comprised of large neoplastic cells showing frequent mitotic activity. Other areas within the tumor demonstrated obvious squamous features.

(R.S.O., unpublished observations). We have recently completed the nucleotide sequence analysis of the RhPV-1 genome and have determined appropriate early-region target sequences usually present in neoplastic tissues. These new primers detected RhPV-1 DNA in the smear of PP100, which had given only a marginal result with the late gene primers (data not shown). These primers also detected RhPV-1 DNA in the smear extracts of PP91 and PP110. However, the sample from the cancer biopsy of PP98, previously found positive by Southern analysis, was negative. We have concluded that significant alterations of the RhPV-1 genome have occurred in this tumor, such that neither set of primers in a PCR assay is useful in detecting RhPV-1 DNA sequences. In several additional samples, positive results were only observed with the PCR analysis due to its remarkable sensitivity. While many of the samples with the weakest signals had no clinical or pathological signs of disease, some of these samples were obtained from abnormal tissues as well. These animals have been infected with RhPV-1 and maintain the RhPV-1 DNA at extremely low levels.

DISCUSSION

We have used several different approaches for the detection of RhPV-1 DNA in a defined population of mating rhesus monkeys. In some cases DNA was detected in an animal by one method, such as *in situ* hybridization or PCR analysis, but not by others due to the limitations of each procedure. In nine animals (29%), RhPV-1 DNA was detected where there had been no evidence of clinical or histopathological disease. By using sensitive DNA detection techniques in various human tissues, parallel studies have found evidence of "latent" viral infection where no clinical disease was evident (13–17). Our results indicate that at least 71% of those animals that had sexual contact with the index male had either clinical, histopathological, or molecular evidence of RhPV-1 infection. One animal had a squamous cell carcinoma localized to the cervix, one had an adenosquamous carcinoma of the endocervix, and eleven (35%) had low-grade warty, dysplastic lesions and/or acetowhitening. Multiple samples decrease the rate of false negatives in pathological and molecular specimens for genital papillomavirus infection. Although we have obtained as many as four samples from some of these animals, it would be safe to say that our results, while closely approaching the true infection rate, are only a lower limit. The presence of false positives was reduced by careful control experiments and by the concordance of the results of the vast majority of histopathological and molecular analyses. None of the female monkeys in this study was the offspring of any other tested female, thus reducing the possibility of a vertical transmission of this virus and, in connection with the other data presented, supporting a sexual mode of transmission for RhPV-1. In each group there was a broad range of ages, some of which could not be exactly determined, as some of the animals were feral. The virgin females were somewhat younger (12.5 yr) than either the animals mated in the cohort (19.2 yr) or those mated outside the cohort (22.8 yr). This is to be expected in a breeding colony such as was examined here. Although age may relate to onset of physical signs of infection due to latency periods, it does not necessarily relate to the ability to detect viral DNA by molecular techniques. The average ages of the animals mated inside or outside of the test cohort are equivalent, as indicated above; yet the groups differ for the presence of RhPV-1 DNA.

RhPV-1 infections in rhesus monkeys offer an excellent model for human oncogenic genital papillomaviruses. All aspects of the disease in these animals including sexual transmissibility, latent infections, and the evolution of a spectrum of benign and malignant neoplastic disease parallel the pattern observed in humans. We have now completed studies showing that RhPV-1 DNA in cooperation with activated Ha-*ras* DNA can transform primary neonatal rat kidney cells after *in vitro* DNA transfection (25). Cells transformed by either RhPV-1 or HPV-16 DNA exhibit anchorage-independent growth and, additionally, could produce tumors in athymic nude mice. Finally, the RhPV-1 and HPV-16 genomes show a high degree of sequence homology throughout the genome (R.S.O., K. LaBresh, and A.F., unpublished work).

These parallel findings in two different species lends great strength to the proposition that certain sexually spread papillomaviruses are intimately involved in the etiology of cervical cancers. The close correlation of these viruses with both disease and its malignant potential, as demonstrated *in vitro*, makes it unlikely that these viruses are simply passengers. Further analysis of the oncogenic nature of RhPV-1 will shed light on malignant progression and offers opportunities for observing disease transmission, performing longitudinal analyses, and studying therapeutic and prophylactic treatments of papillomavirus infections.

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