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Ubiquitous Human Adeno-Associated Virus Type 2 Autonomously Replicates in Differentiating Keratinocytes of a Normal Skin Model

Craig Meyers,* Michael Mane,† Natalia Kokorina,† Samina Alam,* and Paul L. Hermonat†[1]

*Department of Microbiology and Immunology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033; and †Department of Obstetrics and Gynecology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

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Since its discovery in 1966, adeno-associated virus type 2 (AAV) has been described as a helper-dependent parvovirus. However, in this study we demonstrate that AAV undergoes its complete life cycle, devoid of helper viruses or genotoxic agents, in the organotypic epithelial raft tissue culture system, a model of normal skin. AAV progeny production directly correlated with epithelial differentiation, as nondifferentiating keratinocytes were defective for this activity. Large nuclear virus arrays of particles of approximately 26 nm (parvovirus size) were observed in the granular layers of the raft epithelium by electron microscopy. Additionally, dosage-dependent histologic changes, some of which might be interpreted as cytopathology, were induced in the AAV-infected epithelial tissues. These data suggest a new biological model for AAV; that is, AAV is an epithelial-tropic autonomous parvovirus that can alter normal squamous differentiation.

INTRODUCTION

Adeno-associated virus type 2 (AAV) is a small, single-stranded DNA virus of the parvovirus family, genus *Dependovirus* (Berns and Giraud, 1996). Eighty to 90% of the adult human population is seropositive for AAV infection (Mayor *et al.,* 1976). As the name implies, the genus encompasses helper-dependent parvoviruses. Host cells must be co-infected with another virus type (helper) to allow productive AAV replication. Members of the adenovirus and herpes virus families have been well documented to serve as helper viruses for AAV replication (Hoggan *et al.,* 1966; Buller *et al.,* 1981; McPherson *et al.,* 1985). In the absence of a helper virus, AAV maintains itself latently in the host cell by chromosomal integration at a favored site (Cheung *et al.,* 1980; Kotin *et al.,* 1990; Samulski *et al.,* 1992). Thus far there has been no disease state or pathology associated with AAV infection. The ability to maintain a latent state combined with an apparent lack of pathology has made AAV an important candidate as a delivery vehicle for human gene therapy (Hermonat and Muzyczka, 1984; Tratschin *et al.,* 1985; LaFace *et al.,* 1988; Hermonat *et al.,* 1997, 1999; Liu *et al.,* 2000).

The specific role of the helper virus in dependovirus replication is unclear. Under highly unusual circumstances, wild-type AAV has been shown to autonomously replicate in rare immortalized and transformed cell lines when treated with genotoxic agents or when viruses mutated in a *cis*-negative regulatory element were used (Yakobson *et al.,* 1987, 1989; Yalkinoglu *et al.,* 1988; Wang and Srivastava, 1998). Even in these systems replication is low compared to co-infection with a helper virus. However, this low level of autonomous replication suggested that AAV might act as an autonomous parvovirus *in vivo* in specific tissues that have yet to be identified. Recently, several studies have demonstrated that AAV infects stratified squamous epithelium *in vivo* (Georg-Fries *et al.,* 1984; Bantel-Schaal and zur Hausen, 1984; Tobiasch *et al.,* 1994; Han *et al.,* 1996; Malhomme *et al.,* 1997; Friedman-Einat *et al.,* 1997; Walz *et al.,* 1998). We hypothesized that differentiating squamous epithelium may be the favored site for AAV activity. *In vitro* grown organotypic epithelial raft cultures faithfully mimic, both morphologically and physiologically, squamous differentiation and have been shown to be accurate models for normal human epithelium *in vivo* (Asselineau *et al.,* 1986). The raft culture incorporates a combination of keratinocytes layered upon a collagen matrix maintained on a rigid support at the air–liquid interface (Asselineau and Prunieras, 1984; Meyers *et al.,* 1993; Meyers, 1996).

RESULTS

AAV replicates in a normal skin model

Previously, we have demonstrated the efficacy of using this differentiating epithelial tissue system to provide the proper environment for the complete life cycle of the fastidious human papillomavirus *in vitro* (Mayer and Meyers, 1997; Meyers *et al.,* 1992a, b; 1997; Meyers and Wettstein, 1991; Ozbun and Meyers, 1996, 1997a, b; 1998a; b). To investigate AAV activity in differentiating raft

[1] To whom correspondence should be addressed. E-mail: hermonatpaull@exchange.uams.edu.

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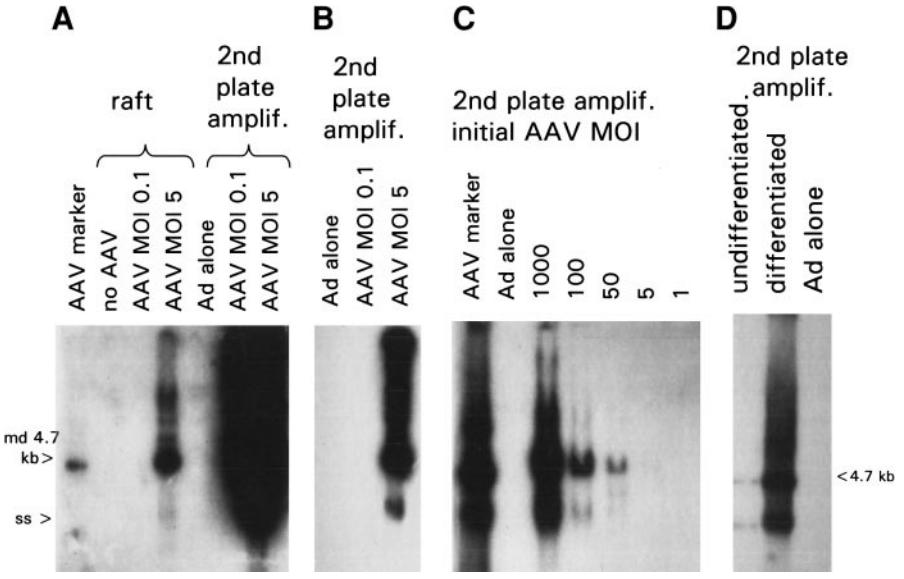


FIG. 1. Southern blot analysis of the raft tissues for replicative AAV DNA and virus production. (A) A 4.7-kb replicative monomer duplex control band of AAV resulting from the infection of the epithelial rafts at a m.o.i. of 10 AAV on adenovirus-infected SW13 cells (AAV marker). Primary epithelial raft tissues mock-infected (no AAV) or infected with m.o.i.s of 0.1 (AAV MOI 0.1) or 5 (AAV MOI 5). Second plate amplification using AAV stocks isolated from mock-infected (Ad alone) raft tissues or isolated from raft tissues initially infected with m.o.i.s of 0.1 (AAV MOI 0.1) or 5 (AAV MOI 5). (B) Shorter exposure of the second plate amplification from A. (C) Second plate amplification using higher initial m.o.i.s of AAV to demonstrate dosage-dependent increases in AAV autonomous replication. This autoradiograph was from a shorter exposure than those for A or B. (D) Second plate amplification of AAV progeny produced in undifferentiated submerged keratinocytes compared to that of the strandard raft procedure (original m.o.i. of 20). The undifferentiated keratinocytes used here were grown and maintained in KSFM (GibcoBRL) supplemented with bovine pituitary extract and epidermal growth factor per company instructions. Putative AAV virus stocks were made from both the raft and the submerged keratinocytes at 5 days postinfection and then used to infect a second plate of adenovirus-infected SW13 cells, as in A.

tissues, primary foreskin keratinocyte monolayers grown on collagen matrices while still submerged in medium were infected with AAV type 2. The following day the cultures were raised to the air–liquid interface (day 0) and allowed to grow and differentiate for 8 days into full-thickness epithelial tissues. Raft tissues were harvested for total cellular DNA and putative AAV stocks. Southern blot analysis revealed autonomous AAV replication when rafts were infected with a multiplicity of infection (m.o.i.) of 5 (Fig. 1A). The monomer duplex replicative form of AAV of 4.7 kb is clearly demonstrated in these cultures. The production of infectious AAV particles was demonstrated by second plate amplification with helper adenovirus coinfection (Figs. 1A and 1B). Higher AAV m.o.i.s were used to demonstrate a dosage-dependent increase in AAV replication (Fig. 1C). Restriction endonuclease digestions were used to characterize the viral DNA and eliminate the possibility that DNA rearrangement or recombination contributed to the ability to autonomously replicate. The expected restriction patterns were observed (data not shown). Additionally, submerged keratinocyte monolayer cultures, which were not raised to the air interface and therefore not allowed to differentiate, were defective for AAV progeny production (Fig. 1E). These results indicate that progeny virus formation was dependent upon squamous differentiation.

To further support *de novo* viral production and observe the kinetics of AAV replication, time-course experiments were undertaken. Epithelial culture tissues were infected with an AAV m.o.i. of 10 at time 0 and tissues were harvested at 12 h and days 1, 2, 3, and 5 postinfection. Total DNA and putative AAV virus stocks were prepared as before. A Southern blot analysis, representative of three such experiments, is shown in Fig. 2A. These experiments revealed surviving input viral DNA at 12 h, which is substantially diminished by day 2 (Fig. 2A). From this analysis, significant *de novo* autonomous DNA replication levels were observed at approximately day 3 and increased modestly with continued differentiation of the host tissue. Similar kinetics were observed for AAV infectious particle production, by second plate adenovirus amplification, with low levels of progeny AAV appearing as early as day 2 and significant levels appearing on day 3 (Fig. 2B). Thus, infection of epithelial tissues by AAV exhibited typical viral infection kinetics with eclipse, logarithmic, and plateau growth phases. These initial experiments were done in mixed foreskin keratinocyte cultures of pooled cells from approximately 40 individuals. To observe if autonomous AAV replication was dependent upon specific individuals or due to general human susceptibility, we assayed keratinocytes from 3 individuals, in triplicate, for their ability to support AAV progeny production (Fig. 2C). AAV progeny formation was seen in the cells from all 3 individuals. However, as the

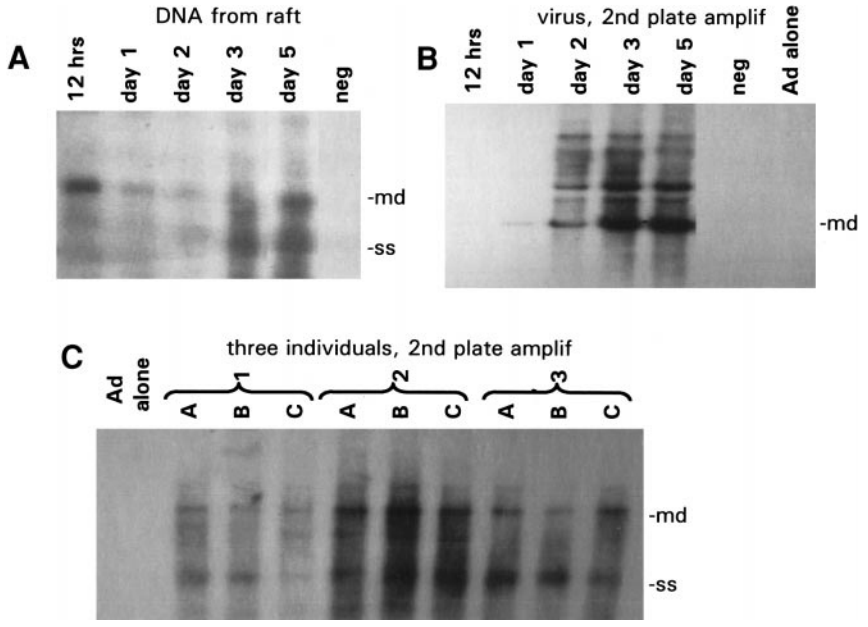


FIG. 2. Representative time course experiment showing *de novo* autonomous AAV replication and virus production. Epithelial raft tissues were infected and generated as before with an AAV m.o.i. of 10. Total DNA and putative AAV virus stocks were prepared and analyzed. Tissues were harvested at 12 h and on days 1, 2, 3, and 5 postinfection. (A) Southern blot measuring AAV DNA levels at increasing times postinfection, representative of three such experiments. Each lane represents 40% of the total DNA isolated from the tissues. The last lane represents mock-infected tissues (neg). The AAV monomer duplex (md) is indicated on the right. (B) Southern blot measuring AAV virus production in the same rafts as in A after second plate amplification in adenovirus-infected SW13 cells. Each lane represents 5% of Hirt-extracted DNA. The last two lanes represent mock-infected tissues (neg) and adenovirus alone second plate amplification, respectively. The AAV monomer duplex is indicated. (C) Epithelial raft tissues generated from three different individuals, each done in triplicate, and each infected at time 0 with an AAV m.o.i. of 20. Putative AAV virus stocks were prepared and amplified in a second plate of SW13 cells as in B and Fig. 1. Each lane represents 5% of Hirt-extracted DNA.

keratinocytes of individual No. 1 showed lower AAV replication levels, some differences cannot be discounted.

No evidence of contaminating helper virus

To further ensure that epithelial differentiation was supporting AAV autonomous replication and not due to a contaminating helper virus, we performed PCR assays to detect the presence of helper viruses. The analysis of papillomaviruses was also included, as there has been one report of their ability to help AAV (Walz *et al.,* 1997). With previously characterized broad-spectrum oligonucleotide primers that targeted adenoviruses, herpes viruses, and papillomaviruses (Allard *et al.,* 1992; Bauer *et al.,* 1991; Crouse *et al.,* 1990), no evidence of a contaminating helper virus was detected (Fig. 3). Importantly, the PCR primers should detect all potential helper viruses that have been or are currently used in laboratories. However, this analysis cannot fully eliminate the possibility that unexpected helper viruses, such as cutaneous HPVs, might be present (this is unlikely, as the keratinocytes originated from neonatal foreskins).

AAV viral arrays are found in the stratum granulosum

Electron microscopy cross sections of AAV-infected primary raft tissues revealed that many cellular nuclei in the stratum granulosum contained substantial virus particle arrays, with the individual virions measuring approximately 26 nm (Fig. 4). Careful examination revealed no other particles of larger size that would indicate the presence of a helper virus. Virions were commonly seen in the uppermost layer of the stratum granulosum in intact nuclei with condensed heterochromatin material. The size and structure are consistent with AAV particles observed in clinical samples and in monolayer-helper virus systems (Walz *et al.,* 1998; Henry *et al.,* 1972; Atchison *et al.,* 1966). Each of these nuclear virus arrays may contain approximately 10^5 virus particles (roughly 40 $\times$ 40 $\times$ 60 particles).

Dosage-dependent histologic changes are seen in the epithelium

AAV replication in monolayer cell culture systems is lytic in the presence of a helper virus. We therefore wanted to investigate the effects of AAV autonomous replication in the three-dimensional architecture of the host epithelial tissue. When infections were performed with a m.o.i. of 1 or 5, morphological abnormalities were observed. These were concentrated in the upper layers, correlating with the presence of intranuclear viral particles. The abnormalities appeared as multinucleated cells, koilocytic-like cells, and areas of apparent cell

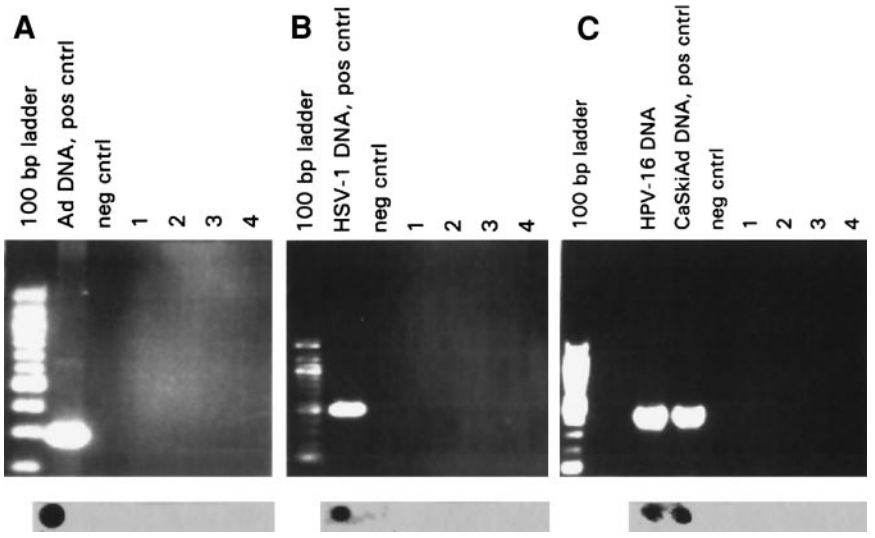


FIG. 3. PCR and dot blot hybridization analysis for contamination by helper virus. Total DNA from four raft tissues derived from the same keratinocyte culture as that used for experiments in Figs. 1, 4, and 5 were analyzed for contaminating adenoviruses, herpesviruses, and papillomaviruses as described under Methodology. The PCR products from each analysis were analyzed by agarose gel electrophoresis with ethidium bromide staining (top) and by dot blot hybridization (bottom). Low stringency conditions were used for washing (1× SSC, 55°C). (A) Broad-spectrum PCR to detect the presence of adenoviruses. ^{32}P-labeled adenovirus type 2 DNA served as the probe. (B) Broad-spectrum PCR to detect the presence of herpesviruses. ^{32}P-labeled HSV-1 DNA served as the probe. (C) Broad-spectrum PCR to detect the presence of papillomaviruses. ^{32}P-labeled HPV-16 DNA served as the probe.

lysis. Figure 5 suggests that AAV infections may not be devoid of pathology.

DISCUSSION

Our investigations strongly suggest that AAV type 2 is an epithelial-tropic autonomous parvovirus whose life cycle is linked to squamous differentiation. In general, AAV has been isolated from stratifying and differentiating tissues of the body such as the cervix, nasopharynx, and anus (Tobiasch *et al.,* 1994; Han *et al.,* 1996; Malhomme *et al.,* 1997; Friedman-Einat *et al.,* 1997; Walz *et al.,* 1998; Blacklow *et al.,* 1967, 1968). Thus, AAV's preference for skin appears similar to that of papillomaviruses. However, differences between the two virus types are also apparent regarding their replication in the skin rafts. HPV replication and progeny formation are greatly enhanced when the skin raft is treated with a protein kinase C inducer, such as TPA (12-*O*-tetradecanoylphorbol 13-acetate), which AAV does not require. Significant levels of AAV DNA replication appear as early as day 3, while HPV requires at least 8 days of squamous differentiation. Finally, the histologic changes in the epithelium resulting from infection also appear to be different, with AAV causing a loss of some cells of the stratum granulosum and higher, presumably by cell lysis. The requirement of host-tissue differentiation for efficient virus replication has been described for many other viruses, such as human papillomavirus (Meyers *et al.,* 1992a, 1997), cytomegalovirus (Weinshenker *et al.,* 1998), Epstein–Barr virus (Crawford and Ando, 1986; Davies *et al.,* 1991; Li *et al.,* 1992), Friend virus (Johnson *et al.,* 1993), human immunodeficiency virus (Cullen and Greene, 1989; Laurence *et al.,* 1990), measles virus (Schneider-Schaulies *et al.,* 1993), polyomavirus (Atencio and Villarreal, 1994), Pichinde virus (Polyak *et al.,* 1991), Rift Valley fever virus (Lewis *et al.,* 1989), and visna virus (Small *et al.,* 1989).

AAV's ability to autonomously replicate in the stratified squamous epithelium is important for our understanding of basic AAV biology and its relationship with human health. Our findings represent a major change in our perception of AAV. This new perception may now include the possibility of a pathogenic role for AAV. Corresponding evidence of clinical pathology needs to be investigated. This discovery also suggests uses for AAV-based gene therapy. In fact, AAV has recently been shown to be efficient in gene delivery to the skin (Braun-Falco *et al.,* 1999). Finally, the organotypic epithelial raft culture system may provide a potential base of a simplified system, without the need for helper virus or parts of helper virus, for generating recombinant AAV virions.

METHODOLOGIES

Viruses, cells, and medium

AAV type 2 and adenovirus type 5 viral stocks were obtained from Dr. Ken Berns. Primary human foreskin keratinocytes were obtained from Clonetics. J2 (Meyers, 1996; Meyers *et al.,* 1993) and SW13 (Hermonat *et al.,* 1997) cells have been described previously. Primary human foreskin keratinocytes (Clonetics) were maintained in keratinocyte SFM medium from GibcoBRL–Life Tech-

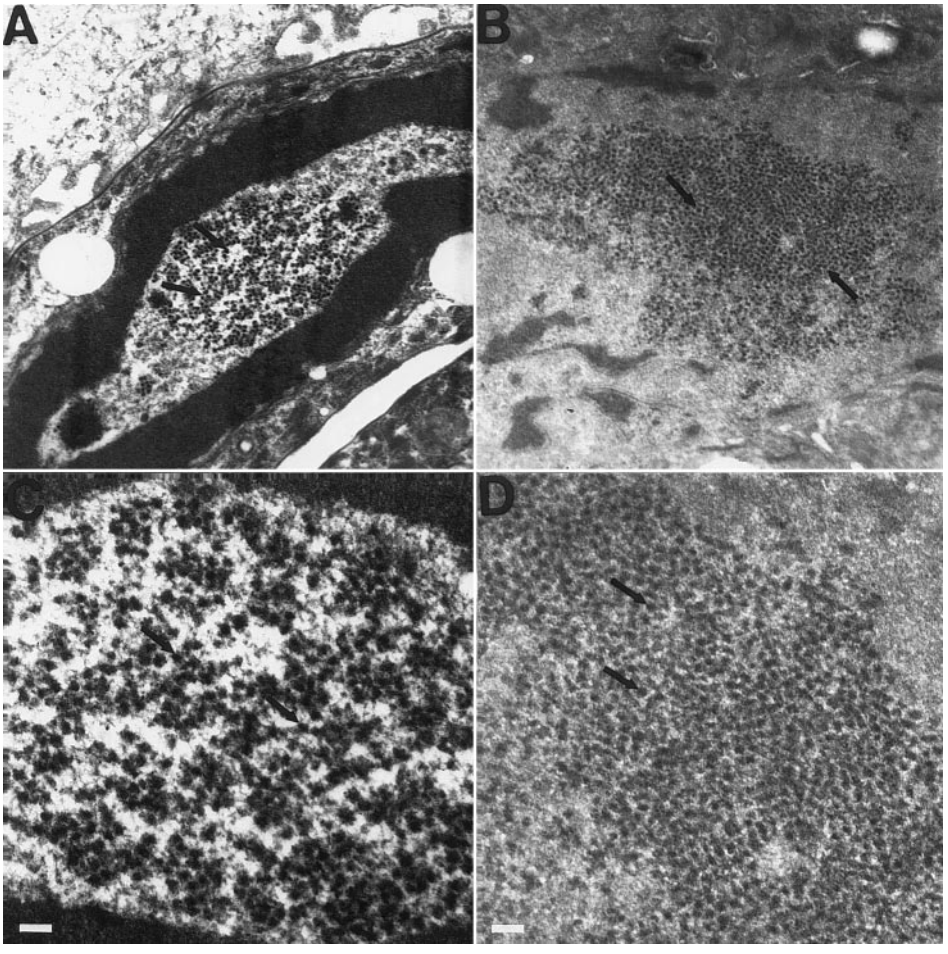


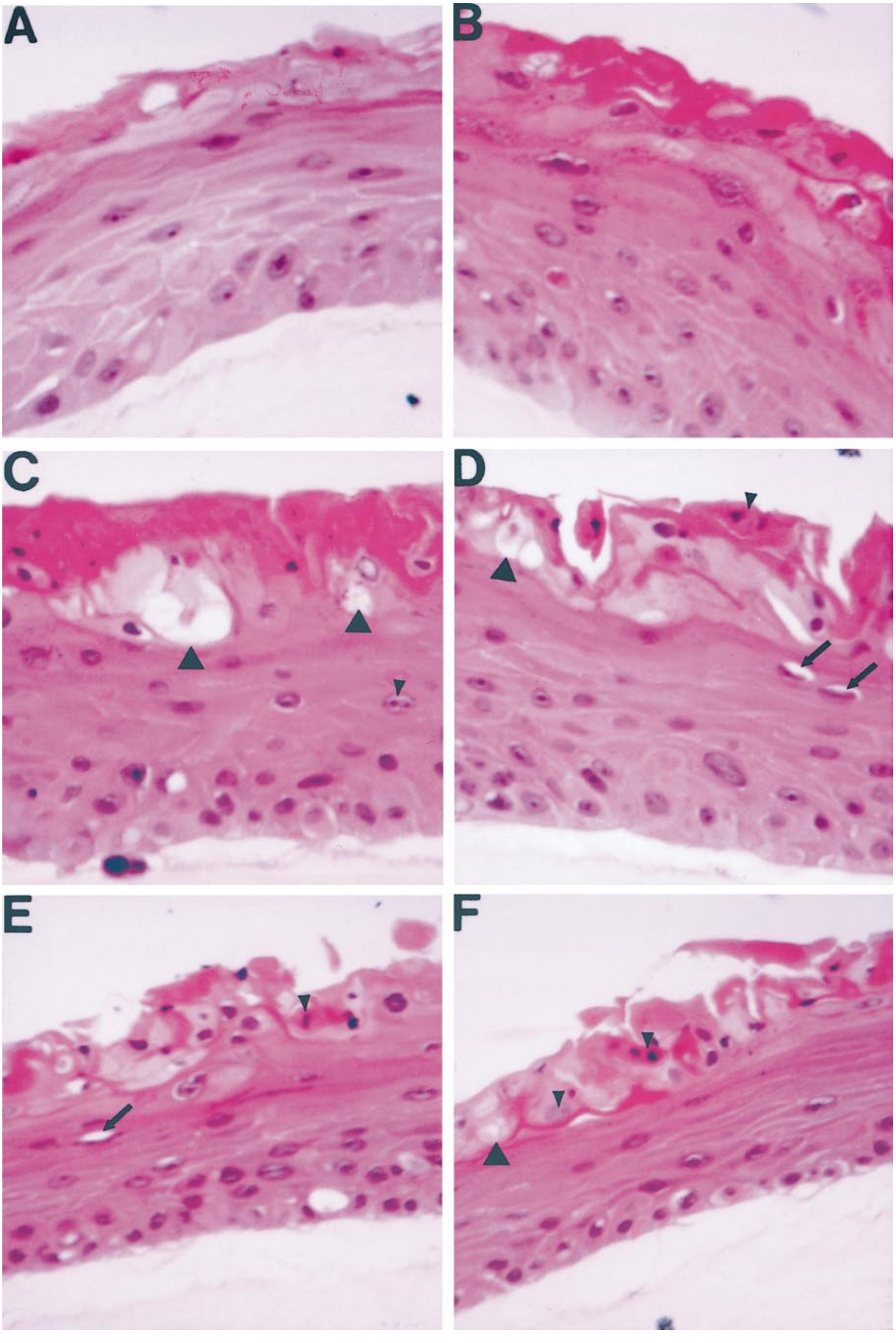
FIG. 4. Examination of primary raft epithelial tissues by electron microscopy for the presence of autonomously replicating AAV. AAV-infected primary epithelial raft tissues were allowed to grow and fully differentiate for 10 days and then were fixed with glutaraldehyde and stained with uranyl acetate. Numerous AAV particles, averaged 26 nm in diameter, were observed in nuclei of the epithelial granular cell layer. (A) A representative nucleus with patches of virions. (B) A second representative nucleus filled with virions. (C and D) Higher magnification views of the virions in A and B (white bars, 50 nm). Arrows point to individual virions.

nologies (Cat. No. 10724-011). Rafts were maintained in E medium, which has been described previously (Meyers, 1996; Meyers *et al.,* 1993). SW13 cells were maintained in Dulbecco's modification of Eagle's medium with 7% fetal bovine serum and antibiotics.

Generation and infection of organotypic epithelial rafts and SW13 cells

Epithelial raft tissues were generated as described previously (Meyers, 1996; Meyers *et al.,* 1993), with the exception that no protein kinase C inducers, such as TPA, were added to the culture medium. Briefly, 2×10^5 keratinocytes were plated onto collagen disks containing J2 cells submerged in E medium and the cells were allowed to adhere for 2 h. Before lifting to the air–liquid interface, medium was removed, AAV type 2 viral stocks were placed on the primary human keratinocyte monolayer in 1 ml of E medium atop the collagen matrix, and cultures were incubated at 37°C for 2 h. At the end of this time, the virus-containing medium was removed and replaced with 5 ml of fresh E medium and the cultures were incubated overnight at 37°C. The next day the raft cultures were raised to the air–liquid interface and allowed to stratify and differentiate as previously described (Meyers, 1996; Meyers *et al.,* 1993). After 12 h/8 days incubation, the raft tissues were then cut in half,

FIG. 5. AAV-induced morphological abnormalities in the host epithelium. AAV-infected primary epithelial raft tissues were allowed to grow and fully differentiate for 10 days and then were fixed with 10% buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. All pictures are at the same magnification, allowing for direct comparisons. (A) Mock-infected. (B) A m.o.i. of 0.1. C and D have m.o.i.s of 1. E and F have m.o.i.s of 5. Arrowheads point to potential suprabasal multinucleated cells, and arrows point to koilocyte-like cells.


A
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and one half was used for DNA isolation and Southern blot analysis for AAV DNA. Measurements of AAV viral production and replication were performed by placing minced tissue into 1 ml of DMEM, freeze thawing 3 ×, filtering through a 0.2-mm filter to remove cellular debris, and treating with DNase I (100 u/ml) for 4 h to degrade unencapsidated DNA. The putative AAV virus stock (100 μl) was then transferred to a 6-cm plate of adenovirus type-5-infected SW13 cells (m.o.i. 5).

Analysis of AAV replication by Southern blot

Total DNA was isolated from the raft tissues. The raft tissue was minced and placed in 500 ml of lysis buffer [5 mM Tris/HCl (pH 7.4), 5 mM EDTA, 0.25 mg/ml proteinase K]. After tissue was digested, the solution was phenol extracted and ethanol precipitated to purify total cellular DNA. For the measurement of AAV progeny formation by second plate amplification assay, after 36 h Hirt DNA was isolated from these second plate amplifications as previously described (Hermonat and Muzyczka, 1984; Hermonat *et al.,* 1997). For Southern blot analysis, 20–40% of total DNA from the infected primary epithelial raft culture tissues (raft) or 10% of the Hirt DNA from the amplified AAV stocks (second plate amplification) was agarose gel electrophoresed, Southern blotted, and probed with ^{32}P-labeled AAV DNA as previously described (Hermonat and Muzyczka, 1984; Hermonat *et al.,* 1997).

Polymerase chain reaction (PCR) and dot blot hybridization analysis for helper virus

PCR and dot blot hybridizations were carried out as described previously (Han *et al.,* 1996) with minor modifications. Briefly, total DNA from raft tissues was used as template and previously characterized broad-spectrum primer sets (Allard *et al.,* 1992; Bauer *et al.,* 1991; Crouse *et al.,* 1990) were used for the amplification of DNA of adenovirus, herpesvirus, and papillomavirus family members. Positive controls consisted of 10^{3-4} copies of the indicated control template DNA (Ad-2, HSV-1, or HPV-16) in 1 μg of SW13 total cellular DNA. Negative controls consisted of the same mix without the sample DNA. The PCR products from each analysis were analyzed both by agarose gel electrophoresis with ethidium bromide staining and by dot blot hybridization. Low stringency conditions were used for washing (1 × SSC, 55°C) to maximize the identification of related viral family members.

Electron microscopic and histologic analyses of skin raft tissues

AAV-infected primary epithelial raft tissues were allowed to grow and fully differentiate for 10 days, fixed with glutaraldehyde, and stained with uranyl acetate, and magnified images were produced by electron microscopy as described previously (30, 32). Size was determined by direct image measurement and division by the indicated magnification. Equivalently treated skin rafts (mock and AAV infected) were fixed with 10% buffered formalin, paraffin-embedded, sectioned, stained with hematoxylin and eosin, and photographed as described previously (Meyers *et al.,* 1992a, 1997).

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