# Analysis of Human Adenovirus Type 19 Associated with Epidemic Keratoconjunctivitis and its Reclassification as Adenovirus Type 64

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**PURPOSE.** Human adenovirus species D type 19 (HAdV-D19) has been associated with epidemic keratoconjunctivitis (EKC), a highly inflammatory infection of the ocular surface. Confusion exists regarding the origins of HAdV-D19. The prototype virus (HAdV-D19p) does not cause EKC, while a virus identified later with the identical serologic determinant is a significant ocular pathogen.

**METHODS.** High throughput genome sequencing and bioinformatics analysis were performed on HAdV-D19p and three HAdV-D19 EKC strains, and compared to the previously sequenced clinical isolate, HAdV-D19 (C) and HAdV-D37. Corneas of C57BL/6J mice were injected with HAdV-D19p, HAdV-D19 (C), or virus-free buffer, and inflammation assessed by clinical examination, flow cytometry, and cytokine ELISA. Confocal microscopy and real-time PCR of infected corneal cell cultures were used to test viral entry.

**RESULTS.** HAdV-D19 (C) and the other clinical EKC isolates showed nearly 100% sequence identity. EKC strains diverged from HAdV-D19p in the penton base, E3, and fiber transcription units. Simplot analysis showed recombination between EKC-associated HAdV-D19 with HAdV-D37, HAdV-D22, and HAdV-D19p, the latter contributing only the hexon gene, the principal serum neutralization determinant. HAdV-D19p induced stromal keratitis in the C57BL/6J mouse, but failed to infect productively human corneal epithelial cells. These data led to retyping of the clinical EKC isolates with a HAdV-D19 hexon gene as HAdV-D64.

**CONCLUSIONS.** HAdV-D19 associated with EKC (HAdV-D64) originated from a recombination between HAdV-D19p, HAdV-D37, and HAdV-D22, and was mischaracterized because of a shared hexon gene. HAdV-D19p is not infectious for corneal epithelial cells, thus explaining the lack of any association with keratitis. (*Invest Ophthalmol Vis Sci.* 2012; 53:2804–2811) DOI:10.1167/iovs.12-9656

Human adenoviruses (HAdV) belong to the genus *Mastadenovirus* within the family *Adenov*iridae, and represent major mucosal pathogens of the ocular, respiratory, genitourinary, and gastrointestinal tracts.[1-3] HAdVs are nonenveloped with an icosahedral capsid, and contain a double-stranded DNA genome. Epidemic keratoconjunctivitis (EKC) is a hyperacute and highly contagious infection of the eye caused principally by HAdVs within species D (HAdV-D), in particular, serotypes HAdV-D8, D19, and D37. More recently, HAdV-D53, D54, and D56 also have been associated with EKC.[4-9] Infection with EKC-associated HAdVs begins with viral entry and replication in ocular surface epithelial cells,[10-13] followed by CXCL-1/8 and CCL-2 expression by infected stromal corneal cells.[14-18] These chemokines bind to negatively charged proteins at the epithelial basement membrane,[19] and induce infiltration of leukocytes into the subepithelial corneal stroma. Subepithelial infiltrates are the sine qua non of EKC and the principal cause of long term visual morbidity.[4,20]

Substantial confusion exists regarding the origins and corneal tropism of HAdV-D19. The prototype virus (HAdV-D19p) was characterized first in 1955,[21] but has not been associated with ocular infection, while a virus identified approximately 20 years later with similar serum neutralization characteristics, but a unique restriction endonuclease digest pattern, is an important etiologic agent of EKC.[22-24] Historically, adenoviral serotypes were distinguished in the laboratory by testing for reactivity of virus-infected cells with virus-specific immune sera.[25] The critical epitope for seroreactivity of the hexon protein is coded for by two adjacent regions on the adenovirus hexon gene, loops 1 and 2, corresponding to hypervariable regions 1–6 and 7, respectively, and representing approximately 2.6% of the genome.[26-29] This general region of the adenovirus hexon gene has been shown to be highly susceptible to homologous recombination between HAdV-Ds and, therefore, is an important source for adenovirus evolution and diversity.[5,9,30-32] Our laboratory previously sequenced and annotated the whole genome of a clinical EKC isolate of HAdV-19, strain C (HAdV-D19 [C]),[33] as well as the whole genome of HAdV-D37,[34] an even more virulent EKC agent. In the present study, we investigated the apparent paradox of HAdV-D19 corneal tropism through whole genome sequencing and bioinformatics analysis of HAdV-D19p and three newly described clinical EKC isolates of HAdV-D19. While all the clinical EKC isolates we sequenced essentially had identical genomes, the HAdV-D19p genome was dissimilar, with only the serologic determinant of the adenovirus hexon gene conserved. Application of in vitro and in vivo models of infection

demonstrated that HAdV-D19p uniquely fails to enter and replicate in human corneal epithelial cells, thus explaining its lack of corneal tropism. EKC-associated viruses characterized previously as HAdV-D19, now retyped as HAdV-D64, represent the product of adenoviral recombination and evolution, and are unique and distinct from HAdV-D19p. HAdV-D64 is yet another example of adenovirus mistyping due to reliance solely on serum neutralization and/or hexon genotyping.[28,35]

## MATERIALS AND METHODS

### Cells and Virus

Human lung carcinoma cell line A549 (CCL-185), a human alveolar epithelial cell line that was shown previously to support HAdV-1 virion production,[36] was obtained from American Type Culture Collection (ATCC, Manassas, VA). Telomerase-immortalized human corneal epithelial (THE) cells were the kind gift of Dr. Jerry Shay (University of Texas, Southwestern Medical Center, Dallas, Texas), and were cultured in defined keratinocyte serum-free medium with supplements (Gibco, Grand Island, New York). Primary human corneal fibroblasts (HCF) were isolated from human donor corneas as described previously.[15] Cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum.

HAdV-D19p was obtained from ATCC. HAdV-D19 (C) used in this study was isolated from a human patient's cornea, as described previously.[14] All other clinical strains of HAdV-D19 described were isolated from patients with EKC. They included HAdV-D19 Miami strain 2, HAdV-D19 Miami strain 3, and HAdV-D19 Pittsburgh strain 88, and were the kind gifts of Dr. Darlene Miller, Bascom Palmer Eye Institute, Miami, FL, and Regis Kowalski, Charles Campbell Laboratory, University of Pittsburgh, PA, respectively. Virus was grown in A549 cells, followed by cesium-chloride gradient purification and dialysis, and then titered in triplicate in A549 cells.

### Whole Genome Sequencing and Analysis

High throughput sequencing of viruses was performed as described previously.[35] Briefly, virus was purified by cesium chloride (CsCl) gradient, dialyzed, and stored at $-80°C$. DNA extraction was accomplished by the addition of proteinase K, phenol:chloroform extraction, and finally ethanol precipitation. Purified DNA was sequenced on a Roche 454 DNA sequencer (Branford, CT) by Operon (Eurofins MWG Operon, Huntsville, AL), to at least 17-fold depth, with an accuracy of greater than 99% (Q20 or better). The sequencing reads were assembled using CLC Genomics Workbench (http://www.clcbio.com/index.php?id=1240), with an N50 average of 5,260. Annotation was performed using a custom annotation engine (Dyer et al, unpublished data) and the Genome Annotation Transfer Utility,[37] with confirmation from NCBI's open reading frame (ORF) finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). Artemis (http://www.sanger.ac.uk/resources/software/artemis/) was used to evaluate the data.[38,39] Open reading frames were BLAST-analyzed against GenBank sequences for confirmation and protein similarity. Splice sites were predicted using the GenScan web server at MIT (http://genes.mit.edu/GENSCAN.html).

Sequences were aligned using the ClustalW[40] option within Molecular Evolutionary Genetics Analysis (MEGA) 4.0.2 (http://www.megasoftware.net/index.html). An online sequence alignment program, mVISTA LAGAN (http://genome.lbl.gov/vista/index.shtml), was used for global pair-wise sequence alignment.[41] Simplot analysis was performed using Simplot 3.5.1 software (http://sray.med.som.jhmi.edu/SCRoftware/simplot/) with a window size of 100 base pairs. Phylogenetic analysis was performed using bootstrap-confirmed neighbor-joining trees (500 replicates), also designed with MEGA 4.0.2. Branches with bootstrap values below 70 (indicative of low confidence) were collapsed.

### Experimental Infections

Animal protocols were approved by the Institutional Animal Care and Use Committee at the Massachusetts Eye and Ear Infirmary, and animals were treated according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. Eight to 12-week-old wild type female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were anesthetized by intramuscular injection of ketamine (85 mg/kg) and xylazine (14 mg/kg). Anesthetic drops (0.5% proparacaine hydrochloride, Alcon, Fort Worth, TX) were applied topically to each eye before injections. A total of 1 µL representing $10^5$ tissue culture infective dose (TCID) of virus, or virus-free dialysis buffer, was injected in the central corneal stroma with a glass micropipette needle fitted with a gas-powered microinjection system (MDI, South Plainfield, NJ) under an ophthalmic surgical microscope (Carl Zeiss Meditec, Inc., Thornwood, NY).[16] At indicated times after injection, the corneas were photographed before euthanasia, which was performed using $CO_2$ inhalation. Corneas were removed and processed for further analysis.

### Flow Cytometry

Corneas were dissected from mouse eyes at the indicated times post infection, and cut into small (1–2 mm diameter) fragments for subsequent digestion with 1 mg/mL collagenase type I and 0.5 mg/mL DNase (Sigma Chemical Co., St. Louis, MO).[5,18] Single cell suspensions were washed twice (300 × g, 5 minutes/wash) in PBS and then incubated on ice for 15 minutes with 2 µL anti-mouse Fc block (BD Pharmingen, San Diego, CA) in a total volume of 100 µL PBS-1% bovine serum albumin (BSA). Following incubation, cells were centrifuged (300 × g, 5 minutes) and resuspended in 5% normal rat serum (Jackson Immuno Research Inc., West Grove, PA) for an additional 15 minutes on ice. Cells were then labeled with 4 µL anti-mouse FITC-conjugated anti-CD45 (clone 30-F11, BD Pharmingen), and incubated in the dark on ice for 30 minutes. Following incubation, the cells were washed 3 times with PBS-1% BSA (300 × g, 5 minutes/wash) and resuspended in PBS containing 1% paraformaldehyde. After overnight fixation at 4°C in the dark, cells were pelleted, resuspended in PBS-1% BSA, and flow cytometry performed using a Cytomics FC500 (Beckman Coulter, Brea, CA) for CD45+ events, representing the numbers of leukocytes present in infected and control corneas.

### ELISA

Mouse corneas were removed at indicated times post infection ($n = 4$/time/group) and flash frozen in liquid nitrogen. Corneas then were sonicated in 400 µL PBS with 1 mM phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, and 10 µg/mL leupeptin (Sigma-Aldrich). The lysates were centrifuged at 10,000 × $g$ for 10 minutes at 4°C, and the supernatants were used for ELISA. Mouse CXCL1 and IL-6 protein quantification was performed with commercially available sandwich ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. Each sample and standard were analyzed in duplicate. The plates were read on a microplate reader and analyzed (Molecular Devices, Sunnyvale, CA).

### Confocal Microscopy

Virus was conjugated with Cy3 dye (GE Healthcare, Piscataway, NJ) as per Leopold et al.[42] A total of 1 mg of Cy3 dye was reconstituted in 1 mL of 0.1 M sodium bicarbonate (pH 9.3). Labeling was performed by conjugating dye to virus at a concentration approximately equal to $10^{12}$ Ad particles/mL, where reconstituted Cy3 dye was 20% of the final solution. The mixture was allowed to incubate for 30 minutes in the dark with gentle mixing every 10 minutes, followed by overnight dialysis to remove the excess Cy3 dye. Cy3-labeled virus preparations were used to infect the cells and imaged by confocal microscopy (Leica TCS SP5, Heidelberg, Germany) at indicated times post infection.
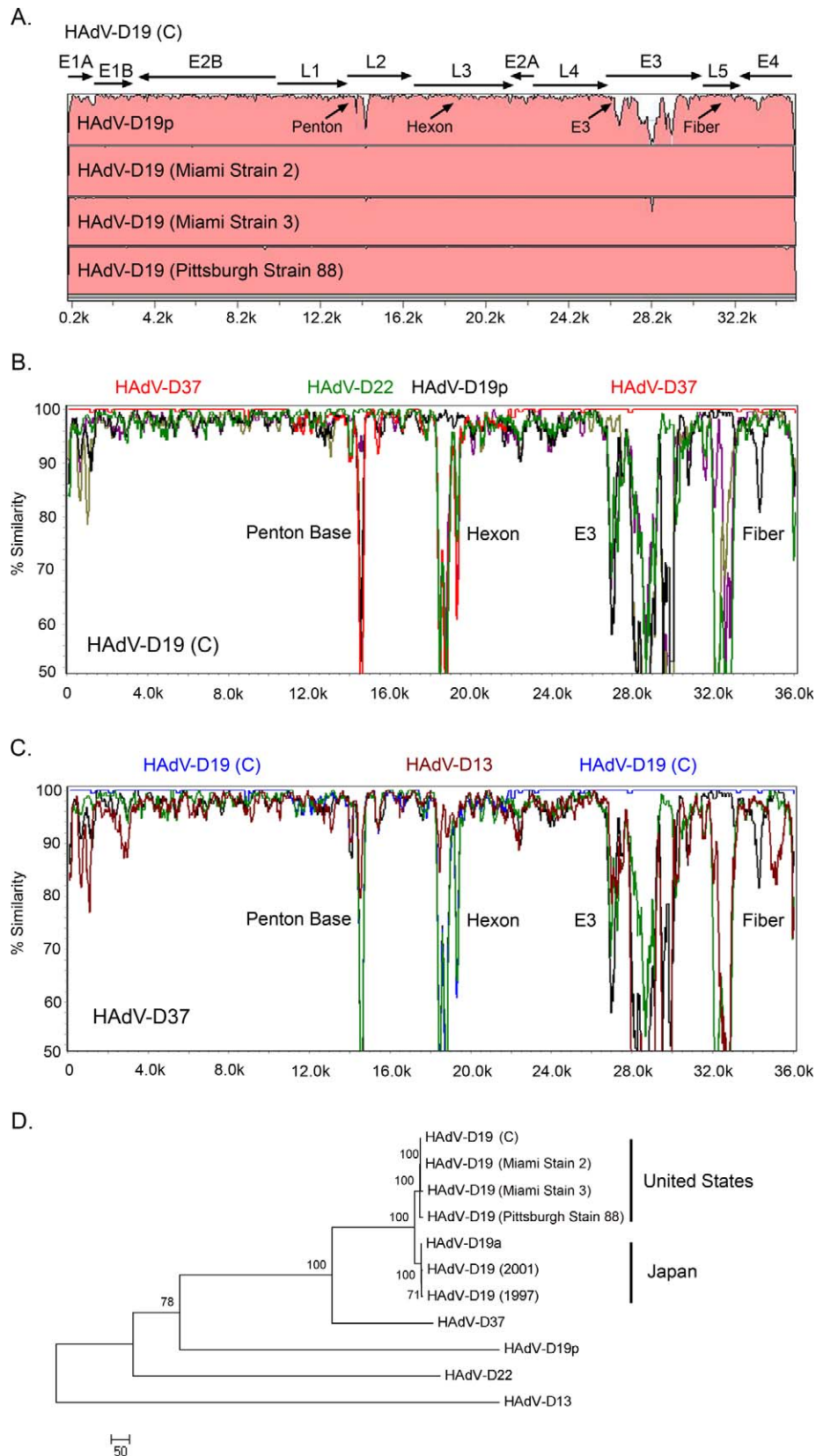
**FIGURE 1.** Genomic and bioinformatics analysis of HAdV-D19. (**A**) Global pairwise alignment using mVISTA LAGAN compares HAdV-D19 (C) with HAdV-D19p and three clinical isolates of HAdV-D19 from patients with EKC. Transcription units are shown above the graph by black arrows relative to their position and orientation in the HAdV genome. Differences across the genome are seen when HAdV-D19 (C) is compared with HAdV-D19p, particularly in the penton base gene and E3 transcription unit. All three clinical EKC isolates were highly similar to HAdV-D19 (C). (**B**) Similarity plot analysis for HAdV-D19 (C) shows evidence for prior recombination with HAdV-D37, HAdV-D22, and HAdV-D19p. Note that only the hexon gene of HAdV-D19 (C) appears to have derived from HAdV-D19p. (**C**) Similarity plot analysis for HAdV-D37, another EKC pathogen, shows recombination

Phalloidin stain (Invitrogen, Eugene, OR) was used to delineate cell cytoplasm, and DAPI (Vector, Burlingame, CA) was used to stain cell nuclei.

## Quantification of Viral Entry by PCR for Viral DNA

Monolayer cell cultures were grown to 95% confluence, and infected at a multiplicity of infection of 20. At indicated times after infection, cells were harvested and DNA separated by phenol chloroform extraction. Quantitative real-time PCR amplification of viral genomic DNA was performed using the 5′ and 3′ primers from a highly conserved region of the E1B gene, TGCTCTGGCCTGCTAGATTC and CTGGCTCCATTTGTCAACCAG, respectively (GenBank acct. no. AB448771), on an ABI Prism 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Results were normalized by standard curve.

## Statistical Analysis

PCR, ELISA and flow cytometry experiments each were performed at least three times. Arithmetic means from three or more experiments were compared by ANOVA with Scheffé multiple comparison test, or Student's *t*-test, using statistical analysis software (SAS institute Inc. Cary, NC). Statistical significance was set at $\alpha < 0.05$.

## RESULTS

### EKC-Associated HAdV-D19 and HAdV-D19p Genomes Share Only the Hexon Gene

The mVISTA Limited Area Global Alignment of Nucleotide (LAGAN) tool[25,29,34,41,43] was used to align and compare paired genomic sequences of HAdV-D19 (C, GenBank acct. no. EF121005), an isolate collected directly from a cornea of a patient with EKC and characterized previously by our group,[33] with HAdV-D19p (GenBank acct. no. JQ326209) and three additional clinical EKC isolates serotyped as HAdV-D19, all of which we sequenced and report herein (GenBank acct. nos. JQ326206, JQ326207, and JQ326208, Fig. 1A). Identity between HAdV-D19 (C) and the other clinical strains associated with EKC spanning their entire genomes was 99.99%. However, HAdV-D19 (C) showed substantial variation from HAdV-D19p in the penton base gene, E3 transcription unit, and to a lesser degree in the fiber gene, with only 95.4% identity between the HAdV-D19 (C) and HAdV-D19p genomes overall. In contrast, the HAdV-D19 (C) and HAdV-D37 genomes were found to be 98.6% identical (data not shown).

We and others have shown previously that viruses in species HAdV-D evolve largely through homologous recombination.[5,9,30–32,44] Therefore, we next performed recombination analysis to determine the evolutionary origins of EKC-associated HAdV-D19 viruses. Simplot analysis showed that HAdV-D19 (C) was a recombinant of HAdV-D37, HAdV-D22, and HAdV-D19p (Fig. 1B). Importantly, the hexon gene, which codes for the serum neutralization epitopes, appeared to derive from HAdV-D19p, thus explaining why HAdV-D19 (C) and HAdV-D19p appear by serum neutralization to be the same virus. Simplot analysis of HAdV-D37 again showed evidence for recombination with HAdV-D19 (C), and also with HAdV-D13, notably in the hexon gene (Fig. 1C). Subsequent phylogenetic analysis of all HAdV-D19 whole genome sequences, including several previously archived from Japan, (GenBank acct. nos:

AB448772, AB448773, and AB448774),[8] showed a close relationship between EKC isolates of HAdV-D19 with the EKC pathogen, HAdV-D37 (GenBank acct. no. DQ900900), and a lesser relationship with HAdV-D19p (Fig. 1D).

### Experimental Adenovirus Keratitis

To understand better why HAdV-D19p has not been associated with EKC, we compared HAdV-D19 (C) to HAdV-D19p in the mouse adenovirus keratitis model.[5,16–18] Virus or virus-free dialysis buffer as a negative control was injected into the corneal stroma of C57BL/6J mouse and the mice observed for clinical evidence of keratitis. Surprisingly, at 4 days after injection, the peak of inflammation in this model,[16] HAdV-D19p appeared more inflammatory than HAdV-D19 (C, Fig. 2A). Buffer-injected mice did not demonstrate clinical signs of inflammation at any time after injection. To compare further the keratitis induced by each virus, infiltrating cells in the infected C57BL/6J mouse corneas were quantified by flow cytometry. Confirming the photographic comparisons at 4 days after injection, CD45+ cells were increased after HAdV-D19 (C) infection as compared to buffer control ($P < 0.05$), but even more so in HAdV-D19p infected corneas (Fig. 2B).

Earlier data using human keratocytes and HAdV-D19 (C) showed upregulation of CXCL8 mRNA within one hour of infection,[15,45,46] and our prior work in the mouse adenovirus keratitis model using HAdV-D37 showed increased levels of CXCL1 and IL-6 protein within 16 hours post infection.[16–18] We compared CXCL1 and IL-6 protein expression in the mouse adenovirus keratitis model by ELISA (Fig. 2C). At 16 hours after injection, CXCL1 protein was significantly upregulated in both HAdV-D19 (C) and HAdV-D19p injected mice as compared to buffer-injected corneas ($P < 0.05$). Il-6 protein expression was also increased by HAdV-D19p ($P < 0.05$), less so by HAdV-D19 (C), suggesting that once in the corneal stroma, HAdV-D19p is at least as or more inflammatory than HAdV-D19 (C).

### Viral Entry into Corneal Cells

Huang et al. showed previously that HAdV-D19p did not enter Chang cells, a conjunctival epithelial cell line, because of a specific amino acid in the fiber knob, the primary ligand for host cell binding.[47] Because of the unexpected finding that HAdV-D19p was pathogenic in the adenovirus keratitis model, which bypasses infection of corneal epithelium, we compared its capacity to infect THE cells and HCF. We first compared cellular entry of Cy3-labeled virus by confocal microscopy, with A549 cells as a positive control (Fig. 3A). At 30 minutes post infection, red fluorescence from HAdV-D19 (C) and HAdV-D19p was seen in the cytoplasm of A549 cells, and to a lesser degree in HCF. Remarkably, even this early post infection, perinuclear red fluorescence was observed upon HAdV-D19 (C) infection of THE cells, while at the same time, no fluorescence was seen with HAdV-D19p infection of THE cells. These data suggest that HAdV-D19 (C) readily infects human corneal epithelium, while HAdV-D19p does not. To confirm these results, we performed quantitative PCR for the presence of adenoviral DNA, using the same cells, and infected with either HAdV-D19 (C) or HAdV-D19p at 1 hour and one week post infection (Fig. 3B). In control testing, the PCR primers equally amplified viral DNA from both viruses (data not shown). In experimental comparisons using A549 cells and

with HAdV-D19 (C), and HAdV-D13, and confirms the plot shown in (**B**). (**D**) Whole genome phylogenetic analysis was performed for newly sequenced and previously archived HAdV-D19 strains, along with other HAdV-D genomes shown to be related closely by similarity plot analysis in (**B**) and (**C**) above. Bootstrap branches with values less than 70 were collapsed. HAdV-D19p claded separately from other HAdV-D19 strains.
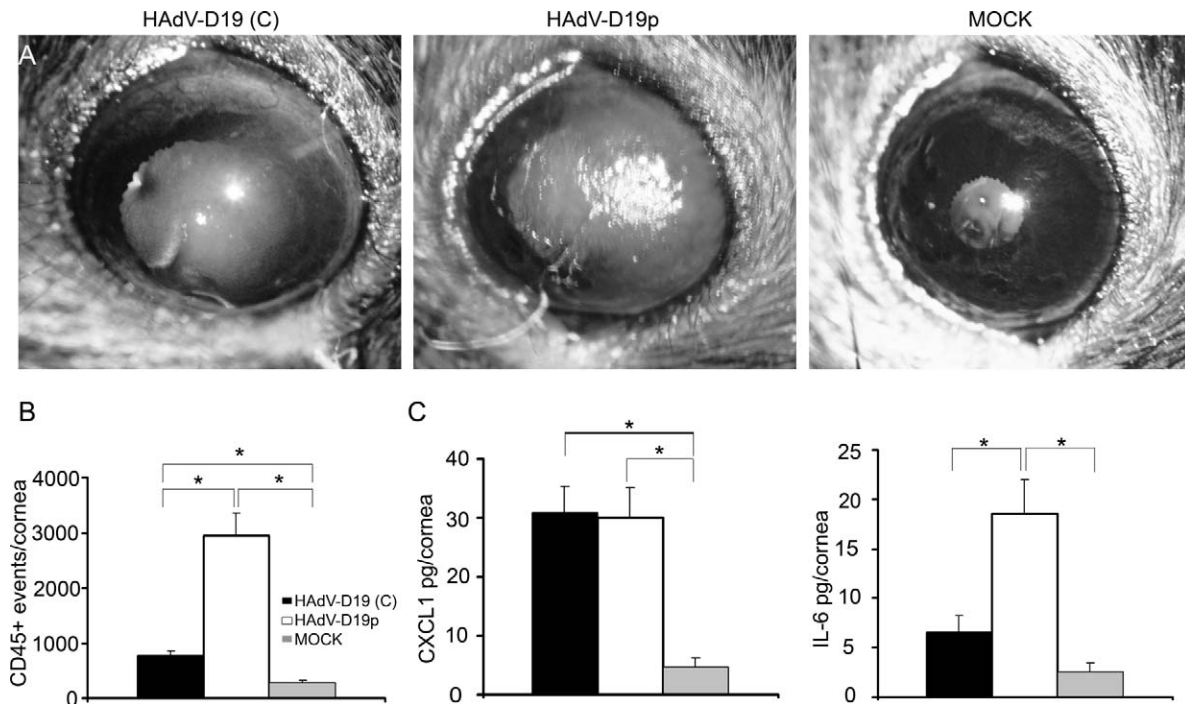
**FIGURE 2.**   HAdV-D19p in the mouse adenovirus keratitis model. (**A**) C57BL/6J mice injected intrastromally with $10^5$ TCID of HAdV-D19 (C), HAdV-D19p, or virus-free dialysis buffer (mock) were compared at 4 days post infection for clinically evident keratitis. Representative photographs ($n = 5$ mice/group) show greater keratitis in the HAdV-D19p infected corneas. No clinically evident inflammation was observed in mock infected corneas. (**B**) Flow cytometric analysis of CD45+ cells in corneas ($n = 4$/group) removed from infected mice at 4 days post infection shows greater leukocyte infiltration in HAdV-D19p infected corneas when compared to HAdV-D19 (C) and mock infected corneas, and greater infiltration in HAdV-D19 (C) infected corneas than mock controls. (**C**) ELISA analysis ($n = 4$ mice/group) at 16 hours post infection shows greater CXCL1 expression in virus infected than in mock infected corneas, with no difference between viruses. However, IL-6 expression was greater in HAdV-D19p infected than HAdV-D19 (C) infected corneas or mock infected controls. (**B**, **C**) Data represent means of three independent experiments $\pm$ SD (*$P < 0.05$, ANOVA).

HCF, both viruses showed increased viral DNA by one week post infection. However, in THE cells, only HAdV-D19 (C) DNA increased, confirming that HAdV-D19p lacks tropism for human corneal epithelium.

## DISCUSSION

In our report, we confirm by bioinformatics analysis as well as in vitro and in vivo experimental studies, that HAdV-D19 (C) and other similar clinical EKC strains are unique from the prototype virus, HAdV-D19p. Our data show clearly that when an EKC isolate demonstrates the serum neutralization properties of HAdV-D19, this should not lead to the conclusion that the type is HAdV-D19. Based on new typing criteria established recently in collaboration with GenBank,[48] and following approval by the Human Adenovirus Working Group (http://hadvwg.gmu.edu/), HAdV-D19 (C) and the other clinical EKC strains we sequenced (Miami strains 2 and 3, and Pittsburgh strain 88) are now reclassified as a [new] human adenovirus, HAdV-D64, based on the next available number for archived HAdVs in GenBank.

Emerging evidence suggests that viruses within species HAdV-D have evolved principally through recombination between closely related types.[5,9,30,31,44] HAdV-D64 represents yet another example of HAdV-D recombination. In this case, recombination conferred new tropism. While HAdV-D64 is associated closely with EKC, the prototype HAdV-D19, which carries the same hexon gene and, therefore, appears similar by serum neutralization tests, is not associated with eye infection. This is similar to HAdV-B55,[28] a respiratory virus that serotypes

as HAdV-B11. Whole genome sequencing and bioinformatics analyses showed that HAdV-B55 carries hexon coding sequence for the serum neutralization epitope of HAdV-B11, a renal tract virus, but the remainder of the genome derives from HAdV-B14, a respiratory virus. In this way, HAdV-B55 may evade herd immunity to HAdV-B14 and, thus, cause acute respiratory distress syndrome in patients who may already be immune to HAdV-B14. Less is known about immunity to EKC associated HAdVs, but immune pressures on viral evolution may be similar.

Genomic sequence data from clinical strains that serotype as HAdV-D19 but cause EKC also have been reported previously by others, and are highly similar to HAdV-D64 and to one another.[8,24,33,49-51] Our data reported demonstrated conclusively that clinical EKC isolates with an HAdV-D19 hexon gene are distinct from HAdV-D19p. Furthermore, we showed that a lack of corneal tropism for the prototype HAdV-D19 likely is due to its inability to infect human corneal epithelium, a requisite first step in adenovirus keratitis. Initial binding of HAdVs to human host cells occurs by an interaction between the fiber knob and several possible host cell receptors, including the Coxsackie-Adenovirus receptor, MHC class I alpha chain, CD46, and GD1a glycan.[52-57] Upon fiber knob binding, a secondary interaction between arginine-glycine-aspartic acid (RGD) in each penton base protein with host cell integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ mediates aggregation of those integrins, autophosphorylation, and downstream intracellular signaling, which ultimately induces endocytosis of the virus into the cell.[55,58,59] Huang et al. previously studied infection of conjunctival cells by the prototype HAdV-D19 in comparison to HAdV-D37, and found that a single amino acid
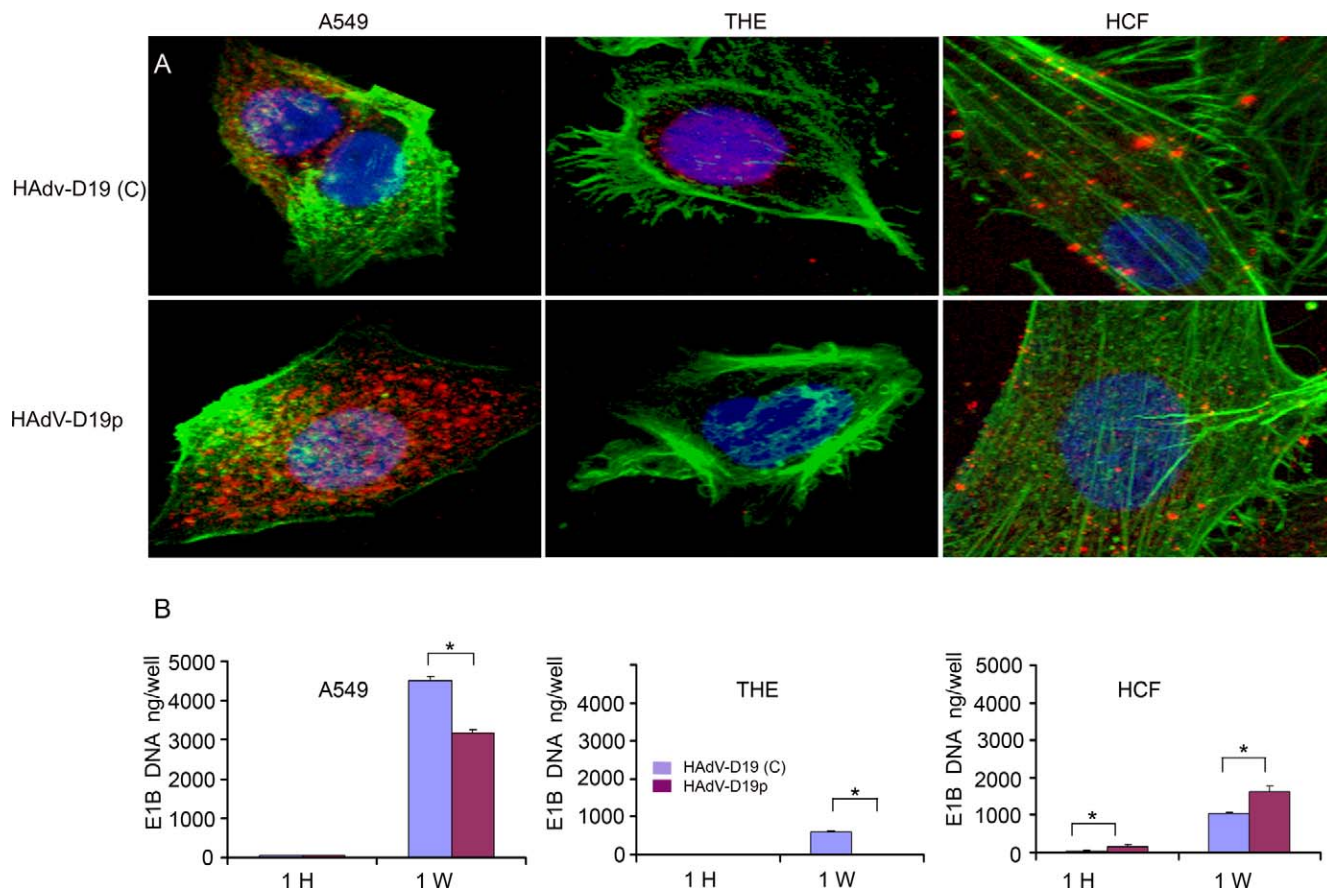
**FIGURE 3.** (**A**) Comparison of infection by Cy3-labeled HAdV-D19 (C) and HAdV-D19p in A549 cells, THE cells, and primary HCF, as seen by confocal microscopy at 30 minutes post infection. Virus appears red, while DAPI stains the cell nuclei blue, and phalloidin stains cellular actin green. Note that HAdV-D19p does not appear to infect THE cells (middle column, lower micrograph) at a time when HAdV-D19 (C) has already reached the perinuclear area (middle column, upper micrograph). (**B**) Assessment of adenoviral DNA by quantitative PCR, using primers against a conserved region of the E1B gene, as a measure of viral internalization and replication at one hour (1 H) and one week (1 W) post infection, respectively ($n = 4$/group). Comparing HAdV-D19 (C) and HAdV-D19p infection of all three cell types, it is apparent that HAdV-D19p fails to replicate (1 week post infection) in THE cells (middle graph), likely due to abortive viral entry. Data represent mean of three separate experiments $\pm$ SD (*$P < 0.05$; Student's *t*-test).

change in the fiber knob accounted for the difference in infectivity.[47] Although we did not study the specific mechanism underlying an absence of binding of HAdV-D19p to corneal epithelial cells, it may be similar to that seen in Chang (conjunctival) cells.

Except for the hypervariable portions of the hexon gene, HAdV-D64 is highly similar to HAdV-D37, suggesting a genomic correlate for corneal tropism. We studied previously the CR-1 gamma open reading frame within the E3 transcription unit, which is identical between HAdV-D64 and HAdV-D37.[60] However, it is more likely that the fiber gene, and in particular the coding region for the fiber knob, represents the critical determinant for corneal tropism. Other gene products as yet uncharacterized also could influence the outcome of infection. Interestingly, in the mouse adenovirus keratitis model, in which corneal epithelial infection is bypassed, HAdV-D19p appeared more inflammatory than HAdV-D64. Although putative genomic correlates for inflammation in the corneal stroma have not been elucidated, HAdV-D22, another virus closely related to the EKC viruses by nucleotide sequence, was not pathogenic in the same mouse model of adenovirus keratitis,[5] suggesting that the capacity of HAdV-D to induce corneal stromal inflammation is complex and still beyond current understanding.

In conclusion, high resolution whole genome data allowed us to determine that EKC-associated HAdV-D19 (HAdV-D64) originated from a recombination between HAdV-D19p, HAdV-D37, and HAdV-D22, and was mischaracterized as HAdV-D19 because of a shared hexon gene. HAdV-D19p is not infectious for corneal epithelial cells, thus explaining its lack of association with keratitis.

## Acknowledgments

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