Published in final edited form as:

FEBS Lett. 2019 December ; 593(24): 3583–3608. doi:10.1002/1873-3468.13693.

Genomic foundations of evolution and ocular pathogenesis in human adenovirus species D

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Abstract

Human adenovirus (HAdV) commonly causes infections of respiratory, gastrointestinal, genitourinary, and ocular surface mucosae. Although most adenovirus eye infections are mild and self-limited, specific viruses within human adenovirus species D (HAdV-D) are associated with epidemic keratoconjunctivitis (EKC), a severe and highly contagious ocular surface infection, which can lead to chronic and/or recurrent, visually disabling keratitis. In this review, we discuss the links between adenovirus ontogeny, genomics, immune responses, and corneal pathogenesis, for those viruses that cause EKC.

Keywords

Conjunctivitis; human adenovirus; epidemic keratoconjunctivitis; genomics; homologous recombination; inflammation; ocular infection; pink eye; tropism; virus evolution

Introduction

Adenoviruses are currently classified by their ancestral host, genomic organization, GC-content, DNA polymerase amino acid sequence distances, and oncogenic properties.[1] There are currently more than 100 recognized human adenovirus (HAdV) genotypes, differentiated by phylogenomics into seven species (A-G). HAdVs contribute to a variety of disease conditions, including respiratory, gastrointestinal, genitourinary, and ocular infections. Given the capacity of adenoviruses to infect and replicate in disparate cell types, adenovirus-derived vectors are commonly used in vaccination and gene therapy applications. Adenovirus research has also played an important role in the study of human biology, where the mechanisms of viral oncogenesis,[2,3] RNA splicing,[4,5] eukaryotic DNA replication,

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[6] antigen presentation, and immune evasion,[7] were first discovered and/or extensively studied using this model agent. Adenovirus is also a pathogen of increasing importance.

Infections likely due to adenovirus were originally described in the 1880's but not formally associated with a specific viral etiology until the early 1950's, following the first identification of the adenovirus. A group of viruses isolated from human adenoids and tonsils, and from pharyngeal and conjunctival secretions obtained from acute infections, was initially characterized as adenoidal-pharyngeal-conjunctival (A.P.C.) viruses.[8-10] In an attempt to isolate "the virus of the common cold", Wallace Rowe and colleagues [11] made in 1953 the seminal isolation of adenovirus from latently infected human adenoids in organotypic culture. Subsequent studies revealed the fundamental physical features of HAdV, its distinct disease associations, and its capacity for persistent infection.

New viral lineages evolve to overcome host selection pressures and to adapt to new hosts, potentially leading to extended tissue tropisms and more virulent infections. HAdV evolves principally by homologous recombination, with a relatively higher recombination propensity within HAdV species D (HAdV-D) than for other species.[12] Not coincidentally, HAdV-D is the most prevalent of the seven species, and to date comprises 73 (71%) of the 103 validated types. The majority of viruses within HAdV-D do not cause clinically significant eye infections. However, a few specific types within HAdV-D are associated with epidemic keratoconjunctivitis (EKC), a severe and highly contagious ocular surface infection. The major EKC pathogens are HAdV-D8, 37, 53, 54, 56, and 64,[10,13-17] with three of these identified in the last decade. Despite the continued emergence of new viruses, the molecular mechanisms that drive recombination and adenovirus ontogeny are not precisely known. Clues from epidemiological investigations, including the emergence of numerous new members of HAdV-D in AIDS patients and those otherwise immunosuppressed,[18-21] suggests that an impaired host-defense either contributes to or is permissive of homologous recombination and the evolution of new adenoviruses.

A major drawback of adenovirus-mediated gene delivery is the activation of type-specific acquired immune responses that result in the loss of vector genomes soon after vector administration.[22] Since the majority of the human population has been exposed and is therefore immune to the more prevalent HAdV types, pre-existing immunity limits their use as adenoviral vectors for vaccination and gene therapy. Additionally, the absence of immunologic memory is important for successful use of adenoviral vectors. Therefore, HAdV types with relatively low seroprevalence, including A31,[23] B35,[24,25] C6,[26-28] D26,[29-31] E4,[32-34] other even less common HAdV types,[35] and simian adenoviruses, [36-38] have attracted attention as potential vector chassis for molecular therapies. In contrast, an innate immune response to adenovirus-based vaccine vectors appears to be critical to development of acquired immunity to the epitope expressed by the transgene.[39] In naturally infected individuals without preexisting exposure and humoral immunity, innate immune mechanisms also typically underlie clinical disease presentations. In EKC, simple binding of the virion to its host target cell is sufficient to initiate inflammatory responses, [40,41] suggesting that initial host immune responses to the physical virion contribute significantly to ocular pathogenesis. Because host immune responses appear important for

adenoviral evolution, disease pathogenesis, and vector implementation, understanding the activation of virus induced inflammatory responses is a paramount undertaking.

In this review, we begin with the story of the discovery of HAdV and its association with ocular infection following a very large outbreak involving more than 10,000 cases during the Second World War. We then focus on the genomics and biology of HAdV evolution with special attention to EKC viral pathogenesis, and illustrate *in vitro* and *in vivo* models of corneal tropism and immune responses. Finally, we highlight the scope of HAdV-D evolution and its impact on ocular pathogenesis.

History of epidemic keratoconjunctivitis – the story of shipyard eye

Adenovirus infection is one of the most common causes of conjunctivitis world-wide. EKC is a particularly severe form, that as can be discerned by its name, includes corneal inflammation. EKC was first described in Austria in 1889,[42] and although suspected over the years to have a viral etiology, was attributed to various other causes due to lack of techniques for virus characterization. The classic triad of clinical signs in EKC consists of membranous conjunctivitis, corneal epithelial erosions, and delayed-onset corneal subepithelial infiltrates (Fig. 1). Other common signs and symptoms of adenovirus conjunctivitis include conjunctival edema, redness, and discharge, eye pain, and blurred vision.[43] In 1930, Wright reported over 900 cases in an epidemic in Madras, India and then experimentally transmitted the disease to unwitting volunteers using filtered conjunctival washings with a filter size too small to pass bacteria or other nonviral pathogens.[44,45]

In 1941, a large outbreak with more than 10,000 cases occurred in the naval shipyards of Pearl Harbor, Hawaii. Thereafter, several thousand cases were seen in San Francisco, with most of the affected persons working in shipyards. Thus, the name “shipyard eye” was tied to the affliction, indicating a suspected occupational association. In 1942, Hogan and Crawford coined the name “epidemic keratoconjunctivitis” after an investigation of 125 cases.[46] However, the name “shipyard eye” remained popular even after other industrial workers were found to be affected.[47] In 1954 at the University of California, San Francisco, a new adenovirus was identified from the eye of a seaman named Trim. This virus was the eighth HAdV isolated with a unique serum neutralization profile, and was named serotype 8, strain Trim,[48] the latter reflecting a naming practice that today would violate patient privacy regulations. The index case presented with acute conjunctivitis, and developed subepithelial corneal opacities typical of EKC. Ten days after the initial contact, the patient’s nurse contracted a similar infection and also developed typical corneal opacities.[48] By 1957, 80% of EKC cases were documented as associated with adenovirus type 8.[49] Mitsui and coworkers [50] subsequently inoculated HAdV-D8 into the eyes of human volunteers who then showed typical clinical and serological responses. HAdV-D8 was re-isolated from the study subjects, thus adding evidence for an etiological role of HAdV-8 in EKC. In Jawetz’s classic description of shipyard eye,[43] he described EKC as an iatrogenic infection spread principally by contamination at ophthalmological clinics; this description was mirrored by the observations of others.[51-53] Today, despite standard infection control protocols mandated by clinic and hospital regulations, adenovirus

outbreaks from ophthalmologic equipment continue to occur in health care settings.[54,55] Extracellular adenovirions are extremely stable and can persist at room temperature for weeks, suggesting that epidemics of adenovirus conjunctivitis and EKC will continue to evade routine prevention measures.

Human adenovirus-disease associations

HAdV infections tend to show species and type specificities. HAdV species B, C and E are associated principally with respiratory disease.[56-58] The illness appears flu-like with symptoms of pneumonia, croup, and bronchitis. HAdV-B, E and several EKC viruses within HAdV-D, also cause self-limited urinary tract infections, presenting as painful passage of blood-stained urine.[59-63] HAdV-F causes gastroenteritis, with severe diarrhea, vomiting, and fever. HAdV-G52, the sole virus within HAdV species G, was also isolated from a patient with gastroenteritis, but the latter association remains unconfirmed [64-66]. Most infections involving HAdV-F occur in young children.[67,68] Adenovirus infections are also a major concern in immunocompromised individuals.[20] In particular, HAdV-A, B, and C have all been associated with infections of allogenic transplant recipients, and with a high mortality rate.[69] HAdV-B, D and E can all cause ocular infections.[70-72]

HAdV infection of the ocular surface presents as one of three main clinical syndromes: simple follicular conjunctivitis, pharyngoconjunctival fever, or EKC. The first two conditions are self-limited and do not result in long term sequelae. However, the keratitis associated with EKC can be chronic and/or recurrent in up to one-third of cases.[73] Follicular conjunctivitis or “ pink eye” is an inflammation of the conjunctiva in which hyperplasia of conjunctival lymphoid follicles, a tertiary lymphoid tissue, is prominent (Fig. 1A). In addition to nonspecific signs of ocular redness and watery discharge, follicular conjunctivitis is characterized by palpable, tender, regional (preauricular) lymphadenopathy. These signs are also common to other acute viral infections of the ocular surface. Adenoviral conjunctivitis is particularly contagious, and involvement in one eye may spread within several days to other eye, to family members, and to coworkers. Acute hemorrhagic conjunctivitis is a variant of acute follicular conjunctivitis, in which subconjunctival hemorrhages are a prominent feature. This infection is caused by HAdV-B11, enterovirus 70, and coxsackie virus A24 variant.[74-76] Although adenovirus infection of the conjunctiva is typically self-limited, serious and potentially fatal consequences can occur in immunocompromised individuals. A recent outbreak of conjunctivitis due to HAdV-B3 infection in a neonatal intensive care unit affected 23 neonates and nine adults, and was found to have originated from use of contaminated ophthalmologic equipment.[54,55] Eleven neonates (48%) and all nine adults had conjunctivitis symptoms, with the same genotype of HAdV-B3 identified as the causal agent across all cases. Four neonates died with respiratory infection complicating their underlying conditions.

Pharyngoconjunctival fever is characterized by the triad of fever, pharyngitis, and acute follicular conjunctivitis, and causes epidemics in school-aged children.[77-79] This infection is caused by HAdV-B and E, and presents as a flu-like illness with conjunctivitis. Although many viral respiratory infections are associated with concomitant follicular conjunctivitis, in pharyngoconjunctival fever, the follicular conjunctivitis is particularly severe.[80,81] The

lacrimal drainage system connects the ocular surface with the nasopharyngeal and respiratory mucosa,[82] possibly accounting for infection at both sites.

The third major adenovirus ocular syndrome, EKC, is the only infection that also involves the cornea.[9,46,48] It is caused mainly by viruses within HAdV-D but is also – less commonly – associated with other adenoviruses, including HAdV-B3, B7, C2, and E4, as well as other as yet unidentified agents.[83,84] EKC is characterized by acute membranous conjunctivitis (Fig. 1B) and delayed-onset subepithelial corneal infiltrates (Fig. 1C). The third component classically associated with the infection, acute corneal epithelial erosions, does not occur in most cases.[73]

Human adenovirus species and type specific features and genomic correlations

Given HAdV species and type specific disease associations, it is reasonable to assume the existence of genomic features that correlate specifically with tropism and virulence. The linear HAdV genome is ~36 kb in length and largely conserved with average nucleotide identities of 84.5-94.8% across species.[85] HAdVs commonly contain nearly 50 known and hypothetical genes.[86,87] The three major capsid proteins (penton base, hexon, and fiber), four minor cement proteins (IIIa, VI, VIII, and IX), six other proteins (V, VII, μ, IVa2, terminal protein, and adenovirus protease), and the viral dsDNA molecule, constitute the physical virion.[88] Adenovirus capsid protein structures share an ancient common ancestor with bacteriophage PRD1, suggesting remote evolutionary relationships.[89,90] Both capsids are organized on a T=25 (pseudo) icosahedral symmetry, the only known models of this arrangement.[91-95] The HAdV hexon protein is closely related to the bacteriophage PRD1 P3 protein; both contain a beta sandwich with four-stranded sheets arranged in a jelly-roll fold.[96] The hexon protein comprises the majority of the capsid with 240 hexon trimers. Twelve penton base pentamers in a ring configuration form the vertices of the icosahedron. A trimeric fiber protein protrudes from each penton base capsomer.

The hexon gene contains seven hypervariable regions (HVRs) comprising (specifying) two adjacent hypervariable loops (HVLs), L1 and L2 on the hexon protein. These HVLs together form the epsilon (ε) determinant, consisting of five serotype-specific B-cell epitopes, that is responsible for serum neutralization.[97-99] Although hexon is the most abundant adenoviral structural protein, it is the trimeric fiber protein that is critical to initial binding of the virion to one of several potential host cell receptors, including the coxsackie adenovirus receptor (CAR), membrane cofactor CD46, and GD1a glycan.[100-105] The fiber protein consists of an N-terminal tail, a central shaft of repeating sequences, and a C-terminal globular knob domain. The three conserved N-terminal tails of the fiber trimer bind to the penton base capsomer.[106] The central shaft domains consist of varying β-repeats that are highly rigid and stable,[107] and connect the N-terminal tail with the c-terminal fiber knob. The fiber knob is the first molecule to interact with the host cell during initial infection, and is also known as the gamma (γ) determinant,[108,109] because antibodies to the fiber knob inhibit hemagglutination of red blood cells in serological assays.[110] Historically, this was used as an accompaniment to typing by serum neutralization, as it permitted the

identification of putatively recombinant viruses in which serum neutralization suggested one type while hemagglutination inhibition pointed to a different type. Such viruses were then referred to as “intertypic”,[19,111-113] a term now obsolete given recent revelations about the role of homologous recombination in HAdV evolution.[114] Structurally, the penton base protein has a basal jelly roll domain similar to that of the hexon,[106] and includes a crucial Arg-Gly-Asp (RGD) motif on one of its two hypervariable loops, HVL2, that interacts with $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrins.[115-117] This interaction in turn induces secondary intracellular signaling that is necessary for viral internalization.[115] Species F enteric HAdVs (F40 and F41), that lack the RGD motif, instead utilize laminin-binding α_2, α_3, and α_6 integrins.[118,119] Both penton base and fiber knob proteins elicit antibody responses that have been shown to have a synergistic effect in neutralizing assays.[120]

A phylogenetic analysis of the highly conserved DNA polymerase gene is shown (Fig. 2) for all 103 currently recognized HAdV genotypes. Of the seven HAdV species, HAdV-D contains the most types, but this may be due to ascertainment bias. However, HAdV-D also shows distinct genomic features that distinguish it from the other species.[12] For DNA polymerase, the types within the HAdV-D clade show a close evolutionary relationship with very short nucleotide distances between types. However, the relatively high conservation among HAdV-D genomes, as exemplified by the DNA polymerase gene, is interrupted by hypervariability at particular regions of the genome, specifically at the three major capsid genes and at three genes within the E3 transcription unit, E3 CR1α, β, and γ (Fig. 3).

By comparison of whole genome sequences, marked differences are seen between species in the major capsid genes.[85] For example, HAdV-A and B penton base genes are largely hypervariable across their entire coding region, while the HAdV-C penton base gene is highly conserved. Uniquely, HAdV-D penton base genes are characterized by general conservation interrupted by two HVRs approximately 450 nucleotides apart. These code for penton base HVL1 and HVL2. HVL2 contains the RGD motif critical to viral internalization,[106] including for those viruses associated with EKC.[117,121] HAdV-D60, which was cultured from a 4 year-old child with bronchiolitis, has a unique deletion of the RGD motif.[122] The HAdV-D60 penton base gene is otherwise highly similar to that of HAdV-D37, a major EKC pathogen. HAdV-D60 productively infected human corneal epithelial cells *in vitro*, but more slowly, and paradoxically induced more severe disease in the mouse model of adenovirus keratitis (discussed below), with increased leukocyte infiltration into the cornea and elevated cytokine expression when compared to HAdV-D37. It may be that the RGD motif is important but dispensable for viral entry, and that delayed viral entry and trafficking [123] are conducive to enhanced host cell innate immune responses, as has been previously suggested.[124]

Homologous recombination of the hexon HVRs appears to be a major mechanism of adenovirus evolution. Hexon recombination between the renal pathogen HAdV-B11 and the respiratory pathogen HAdV-B14 generated the Trojan horse HAdV-B55, a severe respiratory pathogen on a B14 chassis but with the hexon (and associated humoral immune responses) of B11.[125-127] Thus HAdV-B55 avoids herd immunity to HAdV-B14, while retaining its respiratory pathogenicity.[128-131] Recently isolated and genome-characterized in China, HAdV-B55 causes severe acute respiratory disease in immunocompetent adults world-wide,

[132-134] and of sufficient severity to warrant efforts to include it in a vaccine against other fatal respiratory adenoviruses, HAdV-B3 and B7.[135,136] In contrast to marked hypervariability within HVRs of the HAdV-D penton base gene and the entire fiber gene (see below), nucleotide diversity in the HAdV-D hexon gene HVRs is comparatively less in HAdV-Ds as compared to the other HAdV species.[137]

The HAdV fiber gene is entirely hypervariable across all species except HAdV-F, which has two fiber genes. Fiber length is largely due to the variable numbers of β-repeats in the central shaft domain. HADV-B and D fiber genes are shorter and therefore code for shorter fiber proteins than those of HAdV-A or C. Length of the fiber has been inversely correlated with fiber rigidity and hindrance of receptor binding by the fiber knob,[138,139] but shorter fibers are more permissive of docking between penton base RGD and host cell integrins and of subsequent host cell entry without requiring fiber knob binding.[140,141] In comparison to other species, HAdV-D utilizes CD46 and GD1a glycan as primary host cell receptors.[104,105] CD46 also is the major cellular receptor for HAdV-B.[142,143] It was demonstrated that although the motif for CAR binding is present on the HAdV-D37 fiber knob, HAdV-D37's relatively short and more rigid fiber protein provides steric hindrance that restricts interaction with CAR.[139] Binding to CAR may be further impeded by extended, semi-flexible loop structures on the HAdV-D37 fiber knob, as also shown for HAdV-D26 and D48.[144]

Homologous recombination in human adenovirus evolution

Homologous recombination between adenovirus genomes was first discovered in the 1970's by two different groups, with HAdV-C5 and HAdV-A12 as test viruses.[145,146] The first study employed complementation tests in which cells were doubly infected with wild type and temperature sensitive (*ts*) mutants to reveal recombination. The second study utilized co-infection of parental cytopathic virus (cyt^+) and *cyt* mutants;[147] after recombination cytopathogenicity was conferred to the mutants. There is now general recognition of a link between DNA replication and recombination.[148-153] Recombination occurs soon after the initiation of DNA replication, increases with time after infection, extends into the late phase of infection, and takes place at multiple sites within a single genome.[154-156] It has been suggested that the process of adenovirus DNA replication *directly* promotes homologous recombination.[157] Two models for adenovirus recombination were proposed: the Meselson and Radding strand invasion model of recombination, and recombination mediated by complete strand annealing and mismatch repair.[153] However, to date the mechanisms that drive homologous recombination are not completely understood.

It appears from multiple studies that viruses within HAdV-D undergo homologous recombination more frequently than other species, often with important clinical consequences. This also can generate confusion about the infecting strain identity (type) when serology or limited molecular typing methods are used. For example, an outbreak of EKC was reported with a virus that serotyped as HAdV-D22, not otherwise known to be a causative agent for EKC. Limited molecular typing showed hexon identity with D22 but fiber knob identity with HAdV-D8, suggesting a novel recombinant.[158] Subsequent whole genome sequencing demonstrated a genome chassis of HAdV-D37 and D8, with only a

small contribution from HAdV-D22, specifically the region of the hexon gene encoding HVL1 and 2 responsible for seroimmunity. Typed as HAdV-D53,[14] this virus was the second to be acknowledged by the adenovirus community as something beyond a mere "intertypic", a term imputed by many as implying illegitimacy or something uncommon, i.e., not a distinct viral entity. Previous to HAdV-D53, HAdV-D29 was the sole "intertypic" allotted a serotype number by sero-taxonomists.[159] HAdV-D15 and D29 exhibit similar serum neutralization responses but different hemagglutination inhibition reactions, suggesting similar hexon proteins but different fibers. HAdV-D53 was also only the second virus acknowledged as unique based on genomics, after HAdV-G52,[65] which was sufficiently distinct to also justify its designation in a (new) seventh species (G).

To complete the study of HAdV-D53, the D22 genome was also fully sequenced, and analysis of the D22 penton base disclosed high identity between HAdV-D22 penton base HVR1 with that of HAdV-D37, and between HAdV-D22 penton base HVR2 (RGD loop) with that of HAdV-D19.[160] Penton base gene recombination also was then identified in many other viruses within HAdV-D, suggesting a recombination hotspot between the two penton base HVRs.

Further evidence for the importance of homologous recombination as a mechanism of adenovirus evolution arose from whole genome sequencing and analysis of another emergent ocular pathogen, HAdV-D56.[16] HAdV-D56 was associated with a lethal pneumonia in a neonate and keratoconjunctivitis in three caregivers.[161] The viral isolate was initially identified as similar to HAdV-D15/29 by sequencing of hexon HVR1-6 and as HAdV-D9 by hemagglutination inhibition. Subsequent whole genome sequencing and computational analysis demonstrated 100% identity between the virus isolated from the neonate and virus isolated from one of the caregivers with EKC.[16] HAdV-D56 was shown to have a novel penton base HVR1, the penton base HVR2 of HAdV-D9, the hexon gene of HAdV-D15, and the fiber gene of HAdV-D9. A later study identified a fourth virus with the HAdV-D15 hexon gene, HAdV-D69; as with HAdV-D29 and D56, D69 was otherwise disparate for the remainder of its genome.[162]

The prototype HAdV-D19 (HAdV-D19p), originally isolated in 1955,[163] was not an EKC virus. However, a virus isolated from patients with EKC in the 1970's also serotyped as type 19 (HAdV-D19a),[164-166] and was only later whole genome sequenced.[167] Recombination analysis showed that HAdV-D19a contained the penton base gene of HAdV-D22, the hexon gene of HAdV-D19p, and the fiber gene of HAdV-D37, with the majority of genome identical to HAdV-D37 (98.6% overall identity).[17] This led to its reclassification as HAdV-D64. In experimental infections, HAdV-D64 was shown to enter corneal epithelial cells and cause keratitis in the mouse model, while HAdV-D19 did not. HAdV-D64 remains the most common cause of EKC in many parts of the world. These discoveries underscore the importance of whole genome sequencing to establish unique identities of new viruses, and the role of homologous recombination in HAdV-D evolution and EKC pathogenesis.

The HAdV E3 transcription unit codes principally for proteins that mediate immune evasion by the virus,[168-171] including down-regulation of interferon signaling, interference with tumor necrosis factor-mediated cytotoxicity, and prevention of antigen presentation to

cytotoxic T lymphocytes. Homologous recombination within the HAdV-D E3 transcription unit was identified as common and specific to the E3 CR1α, CR1β, and CR1γ genes.[172] A notable instance of HAdV-D E3 recombination was identified in a mixed infection with two different viruses containing same hexon, HAdV-D29 and HAdV-D30, in which a single recombination event just prior to the E3 transcription unit resulted in a novel virus, HAdV-D63, with the fiber gene of D29.[173] Remarkably, every unique HAdV-D analyzed to date shows evidence for at least two recombination events.[12] It is noteworthy that the major recombination sites within HAdV-D all code for proteins that interact with the immune system, either by direct interaction between the major capsid proteins and antibodies and/or T cells,[174] or indirectly through modulation of immune functions, as with the E3 encoded proteins. These observations suggest that recombination is an important means of adenoviral adaptation to host immune pressures. Further, although the current typing methodology accepted by GenBank defines a new HAdV type by molecular typing of the three major capsid genes, the addition of E3 identities would undoubtedly lead to an even more granular differentiation between HAdV genomes.

Historically, homologous recombination was thought to occur within HAdV species but not between them. However, interspecies recombination has been demonstrated for HAdV-E4, [175] the only member of species E. The HAdV-E4 genome was shown to have laterally transferred its hexon gene to the genome of HAdV-B16. The HAdV-E4 genome is highly similar to a simian (chimpanzee) adenovirus (SAdV)-E26. Subsequently, two highly similar isolates of another virus, SAdV-B35, one isolated from a chimpanzee and the other from a bonobo, were shown to share genomic regions with SAdV-B21 and SAdV-B27, but also had regions of high similarity to HAdV-B21 and HAdV-B16, suggesting prior zoonotic or anthroponotic transmission with subsequent recombination events.[176] Since both HAdV-B21 and -B16 are human respiratory pathogens, it was suggested that SAdV-B35 could be a nascent human respiratory pathogen. This idea proved prescient when a recent analysis of an adenovirus originally isolated from a fatal respiratory infection in a 6 year-old boy in 1965 revealed a virus genome nearly identical to SAdV-B35.[177] This inter-species recombinant was typed as HAdV-B76. In contrast, no HAdV-D genome has shown evidence for recombination with other HAdV species or with adenoviruses from simian hosts.

Molecular mechanisms underlying HAdV-D recombination in the penton base gene

As discussed above, binding of the RGD motif on penton base HVL2 to host cell integrins mediates intracellular signaling and viral internalization. Using a mouse model (described below), injection of a 15-mer synthetic RGD-containing peptide into the cornea simultaneous to viral infection competitively inhibited HAdV-D37 keratitis, while injection of a RGD-replaced control peptide had no effect.[178] This suggested a vital role for penton base RGD interaction with corneal cells as a determinant of corneal pathogenesis in EKC. In contrast, HAdV-D60 with a natural deletion of the RGD loop induced significantly more inflammation in the mouse keratitis model.[122] However, the delayed entry and slower trafficking of HAdV-D60 as compared to that of HAdV-D37 may have initiated a more sustained intracellular signaling cascade, as has been suggested to be important to chemokine gene expression induced by adenovirus infection.[179] Regardless, the relatively

high frequency of penton base recombination in HAdV-D suggests substantial host pressure and an important role for the HAdV-D penton base protein in viral pathogenesis.

The penton base gene HVRs 1 and 2, which code respectively for penton base protein HVLs 1 and 2, are separated by ~ 450 nucleotides. These have been shown to recombine independently of one another,[85,160] suggesting the possibility that a nucleotide recombination signal or motif might exist between HVR1 and HVR2 that facilitates homologous recombination. In several bacterial species and in bacteriophage lambda, the crossover hot spot instigator or “Chi” nucleotide sequence mediates homologous recombination using bacterial Rec enzymes.[180-183] In brief, the RecBCD protein complex with both DNA-unwinding (helicase) and DNA hydrolysis (nuclease) activities initiates recombination by binding and unwinding DNA. Upon Chi-site recognition, the RecBCD nicks the Chi-strand and then RecA enzyme is loaded to create an ssDNA-protein filament, which invades homologous dsDNA, leading to homologous recombination. [184-186] This Chi site-mediated recombination fits the aforementioned Meselson and Radding strand invasion model for adenovirus recombination.[153] Remarkably, Chi-like nucleotide sequences were identified in HAdV-Ds immediately adjacent to the 5’ side of penton base HVR2.[187] When co-infected in the presence of an *E. Coli* lysate containing Rec proteins, HAdV-Ds showed increased homologous recombination; with RecA depletion or with a RecA mutant, recombination was reduced. These data suggest that HAdV-D evolution might be facilitated by bacterial flora present at mucosal surfaces, particularly in the gastrointestinal and respiratory tracts, where persistence and co-infection by adenoviruses are common.[188,189]

HAdV-D genomic signatures – determinants of corneal tropism

HAdV-D15, D29, D56, and D69 all have highly similar hexon genes with otherwise disparate genomes.[162] When injected into the mouse cornea, all four types induced keratitis, but only HAdV-D56, the sole member of this group associated with EKC, was shown to infect human corneal epithelial cells and human corneal fibroblasts *in vitro*. Therefore, although the presence of virions in the corneal stroma, even from disparate HAdV-D types, will variably induce inflammation, tropism of specific HAdV-Ds for the cornea is clearly not determined by the hexon protein. The fiber knob is the specific ligand on the adenovirus that first engages the host target cell, and its binding is currently a prime target for experimental therapies against EKC.[190-193] Previous data showed a single amino acid substitution in HAdV-D19 fiber knob, from glutamine to lysine at amino acid 240 (numbered according to HAdV-D37), conferred binding to the immortalized Chang C human conjunctival cell line (now known to be HeLa cell contaminated).[194] A reverse substitution in the HAdV-D37 fiber knob abrogated binding. Given its importance to host cell receptor binding, a comprehensive phylogenetic analysis of HAdV-D fiber knobs revealed a pattern wherein the six EKC-associated viruses form a distinct “EKC clade” that was not seen for other genomic regions.[195] Detailed amino acid alignments showed that a lysine or alanine residue at position 240 differentiates EKC from non-EKC viruses. The ratio of nonsynonymous substitutions (dN) to synonymous substitutions (dS) identified amino acid 240 to be under positive selection (dN/dS>1). HAdV-Ds with this amino acid residue also readily entered human corneal epithelial cells *in vitro*. Productive infection of ocular

surface cells including the corneal epithelium is presumably requisite to the development of EKC. Therefore, selection pressure in the HAdV fiber knob is a key determinant of corneal epithelial cell tropism.

Prediction of the next EKC pathogen

Given that the first step in corneal infection by adenovirus is fiber knob binding to its receptor on corneal epithelial cells, analysis of fiber knob binding motifs might be useful to predict which HAdVs can infect the cornea and cause EKC, even in the absence of a previously known clinical association. With only one exception, the known EKC types within HAdV-D contain the fiber gene of either HAdV-D8 or D37 (Fig. 4A). The sole exception is HAdV-D56, which through recombination acquired the fiber gene of HAdV-D9, [16] the latter not known to be a cause of EKC. Using HAdV-D8 and D37 as paradigm EKC pathogens, it was suggested that a lysine or alanine at amino acid 240 was necessary for corneal epithelial tropism;[195] HAdV-D56 (and D9) has an alanine at this site. Real-world confirmation of the association between the HAdV-D8 and D37 fiber knobs and corneal tropism, derives from analysis of HAdV-D85,[196] a recently characterized virus that caused EKC, and HAdV-D82, which was suggested to be an emerging EKC pathogen in one report. [197] Remarkably, HAdV-D85 has the fiber gene of D8, while HAdV-D82 has the fiber gene of D37. However, there must be other factors associated with the potential to cause EKC, as although HAdV-D9 readily enters human corneal epithelial cells *in vitro*, it has not been shown to cause EKC. Interestingly, the HAdV-D8 Trim strain housed at the American Type Culture Collection (ATCC, Manassas, VA) was found to be contaminated with HAdV-D9 and D10,[198] and both D8 and D9 were isolated together from a patient with ocular infection (not specifically characterized as EKC).[198,199] These data raise the possibility that HAdV-D9 might, in certain circumstances, cause EKC. Newer HAdV-D isolates with the HAdV-D9 fiber gene, not known to be associated with EKC, but predicted as potential EKC pathogens, are listed in Fig. 4B.

Modeling host immune responses in EKC

Although the current emphasis in drug discovery for ocular surface infections by adenoviruses has been focused on the identification of antiviral drugs, managing host immune responses to the infection is likely critical to control of clinical symptoms. In this section, we briefly report findings from basic studies on adenovirus-induced cell signaling using human corneal cells. We then describe two novel models of ocular adenovirus infection: an *in vitro* model of the human cornea in four dimensions, and an *in vivo* model of adenovirus keratitis in the mouse. Experiments performed in these two models generally confirm prior observations from those performed on monolayer cell culture of human corneal cells.

Cell culture models of HAdV-D37 and D64 infection

HAdVs can be readily cultured from the corneal epithelium of infected patients.[200] However, studies of mRNA and protein expression by infected cells suggested that human keratocytes, which make up about 85% of all cells within the underlying corneal stroma, produce significantly more chemokines than human corneal epithelial cells.[41] In infected

keratocytes, gene expression of CXCL8 and CCL2, key chemoattractants for neutrophils and monocytes respectively, was evident within 1-2 hours after HAdV-D64 infection,[201] prior to expression of E1A.[202] Induction of proinflammatory gene expression in HAdV-D64 infected human keratocytes is driven by a signaling cascade involving protein kinase C, Src, and focal adhesion kinase, with downstream activation of several mitogen-activated protein kinases (MAPKs) to differentially drive NFκB activation and nuclear translocation. [179,202-207] Both the duration and timing of upstream signaling determine the specifics of NFκB dimerization and transcriptional activity. Furthermore, upstream activation of the phosphoinositide 3-kinase/Akt pathway stabilizes the cell against apoptotic cell death and enables viral replication.[208] It appears that these signaling pathways are part of the same signaling network that mediates viral internalization in this cell type.[202,206,208-210]

Human cornea facsimile model of adenovirus keratitis

The human cornea, from anterior to posterior, consists of 1) a 5-cell layered, 50-micron thick, corneal epithelium, 2) a collagenous stroma of 500 to 1000 microns thickness, intermixed with keratocytes and a lesser population of bone marrow derived cells, mostly macrophages, 3) Descemet's membrane and 4) a monolayered endothelium. Keratocytes assume a fibroblast phenotype in the presence of serum and are then referred as human corneal fibroblasts (HCFs).[41,211] An *in vitro* three-dimensional culture model of the human cornea was originally developed to study collagen degradation by keratocytes.[212] A similar system using primary, cultured, human keratocytes embedded in type I collagen and overlaid onto a tissue culture transwell insert was exploited to show the induction of CXCL8 upon HAdV-D64 infection.[41] The initial iteration of this model showed diffuse CXCL8 reactivity in the collagenous stroma that did not explain the subepithelial localization of infiltrates seen in human adenovirus keratitis. Because CXCL8 stably binds basement membranes,[213] the model was further adapted by overlaying Matrigel® on the upper surface of the collagen (Fig. 5A). Matrigel is a gelatinous protein complex secreted by cultured mouse sarcoma cells, containing many components of epithelial basement membrane, and previously shown to bind interferon gamma.[214] Immunohistochemistry performed on facsimiles overlaid with Matrigel and after overnight infection with HAdV-D37 showed foci of CXCL8 bound to the Matrigel, and colocalized to heparan sulfate.[215] Heparan sulfate is a component of corneal epithelial basement membrane [216] also present in Matrigel, and expected to bind to CXCL8 because of its negative charge.[213] This model was further extended by the addition of human peripheral blood leukocytes (PBLs) beneath the insert. After overnight infection, and just one hour after placing PBLs beneath the insert, myeloperoxidase positive neutrophils had migrated upwards against gravity to establish sub-Matrigel foci of inflammatory cells (Fig. 5B), similar to subepithelial corneal infiltrates observed in infected human patients. Pretreatment with chemical inhibitors of Src (PP2) and p38 MAPK (SB203580) completely blocked the formation of the neutrophil infiltrates. It was also shown that the virus alone in the absence of keratocytes was insufficient to induce leukocyte migration (Fig. 5C). Therefore, the human cornea facsimile model of adenovirus keratitis recapitulates the formation of subepithelial infiltrates in HAdV-D infected human cornea and confirms a role for corneal cell signaling in corneal inflammation.

Mouse model of adenovirus keratitis

Animal models of adenovirus keratoconjunctivitis are limited by species-specific tropisms of the virus. The cotton rat (*Sigmodon hispidus*) (Fig. 6A),[217,218] New Zealand White rabbit (*Oryctolagus cuniculus*) (Fig. 6B),[219-221] and Hollander rabbit (*Oryctolagus cuniculus*) (Fig. 6C),[222] have all been used in experimental studies of ocular surface infection by adenoviruses. All require both topical and intrastromal (corneal) viral inoculation. Cotton rats in particular are difficult to breed and handle, and although they develop keratitis upon HAdV-D8 inoculation, these animals have not proven tractable for immune studies. HAdV-Ds do not replicate in rabbit models. Complicating this, antibodies to rabbit immune markers are only sparsely available. With rare exception,[217] most studies in rabbits have focused on trials of antiviral agents,[219,221,223-227] rather than studies of immune responses. These limit the use of these models in studies of adenoviral immunopathogenesis. Importantly though, it was shown in the rabbit model that intrastromal inoculation of HAdV-B14 could induce keratitis despite an absence of viral replication.[228]

HAdV does not replicate in mice, but Eggerding and coworkers showed that early viral genes are transcribed in permissive mouse cells,[229] consistent with viral attachment and entry. Ginsberg and colleagues infected the lungs of C57Bl/6N mice with HAdV-C5 to study adenovirus pneumonia – they showed that HAdV-C5 does not replicate in the murine lung but that infection with very high titers of virus (10^{10} plaque forming units) can induce substantial inflammation.[230] The latter observation was applied to develop a mouse model of HAdV-D37 keratitis in which virus enters corneal stromal cells, early viral gene expression is induced, and clinical keratitis develops similar to human patients; this most optimally occurred in female C57Bl/6J mice.[231,232] Corneal injections are performed with heat-pulled glass micro-pipette needles, similar to needles used for single cell injections, and at least 10^5 $TCID_{50}$ of purified, endotoxin free HAdV-D37 is required to reproducibly induce clinically evident keratitis (Fig. 7A). Successful corneal stromal injection is evident by transient whitening of the corneal stroma. Stromal inflammation is evident beginning at 1 day post-infection, and peaks at 4 days. Ultrastructural studies subsequently established robust viral entry into stromal keratocytes (Fig. 7B). However, capsids appeared to “stack up” in the cells, suggesting failure of capsid disassembly and a scarcity of nuclear entry.[233] It was postulated that failure of HAdV-D37 to replicate in the mouse cornea might be due to disordered intracellular trafficking or difficulty interacting with key nuclear pore proteins.[210] Inflammation is initially diffuse and then clears, but at approximately 6 weeks post-injection, a subset of mice develop corneal subepithelial infiltrates similar to patterns for recurrent adenovirus keratitis in human patients (Fig. 7C).

Using this model, it was shown that neutrophils constitute the first wave of infiltrating immune cells upon corneal stromal infection, and that this is due to CXCL1 expression by infected corneal stromal cells.[234] The cells productive of CXCL1 were presumed to be keratocytes although myeloid lineage cells also are present in small numbers in the corneal stroma.[235] It was then demonstrated that the HAdV-D37 capsid, specifically the penton base RGD, was a major molecular pattern responsible for the initial innate immune response to infection.[178] Activation of MyD88 and Src were identified as key to the activation of downstream proinflammatory mediators.[236]

Viral persistence, host immune suppression, and the evolution of new human adenovirus D types

Homologous recombination between HAdVs requires co-infection of the same cell by two viruses with significant homology. After infection, even at disparate physical sites, HAdV infection persists in nasopharyngeal and gastrointestinal lymphoid tissues, with continued viral shedding for months to years after the initial infection.[11,237-245] Adenovirus persistence allows continuous production of progeny virus over an extended period, albeit at lower levels than during acute infection.[246] Viral persistence increases the odds of co-infection, and indirectly promotes homologous recombination and viral evolution. A study of HAdV persistence in human T-lymphocyte cell lines provided evidence for persistence being type-dependent, suggesting there are viral genetic determinants of HAdV persistence. [247]

In 1995, Khoo and coworkers estimated the 1-year actuarial risk of adenovirus infection in persons infected with HIV to be 28%.[199] Multiple, previously unrecognized HAdV-Ds were discovered during the global AIDS epidemic, including HAdV-D43-51, D58, D59, D62, and D75.[18,248-258] In addition, numerous reports detail previously identified viruses that were found to infect AIDs patients.[21,199,248,251,258] Other novel, so-called “intertypic” HAdV-D recombinants were identified from patients immunosuppressed due to other causes.[18,19] These observations suggest that immune compromise may offer a platform for the emergence of new HAdV-D types by recombination. Using a persistent adenovirus infection model *in vitro*, Zheng and colleagues demonstrated that interferon gamma inhibits the association between the transactivator GABP with the HAdV EIA enhancer region, suppressing E1A expression,[259] and promoting persistent infection. In parallel experiments, blockade of interferon signaling and removal of E1A repression led to a transition from a persistent to a lytic infection. Relevant to these findings, HIV infected individuals may show a dramatic decline in interferon gamma levels at late-stages of infection.[260-262] One might predict that immunosuppressive drugs that function by depleting interferon gamma producing T and NK lymphocytes, or directly repressing cytokine signaling pathways, in addition to their association with disseminated adenovirus infections,[245] could promote the emergence of new HAdVs through enhancement of viral DNA replication. These ideas are consistent with the notion that loss of host interferon responses leads to HAdV reactivation and dissemination in immunocompromised persons, [263] and indirectly promotes the emergence of new HAdVs through homologous recombination.

Conclusions

In this review, we described the original association of HAdV with severe eye infections, identified special aspects of HAdV genomics, provided observations indicating recombination as a driving force for evolution specific to those viruses that cause severe keratoconjunctivitis, presented two unique experimental models of HAdV keratitis, and showed how the intersection of HAdV genomics and biology can illuminate concepts in HAdV ocular pathogenesis and evolution.

Acknowledgments

Funding

This work was funded by National Institutes of Health grants EY013124, EY021558, and EY014104, a Senior Scientific Investigator Award grant (to JC) from Research to Prevent Blindness, Inc., New York, NY, and the Massachusetts Lions Eye Research Fund.

Abbreviations

CXCL8	interleukin 8
CCL2	monocyte chemoattractant protein-1
CsCl	ceasium chloride
EKC	epidemic keratoconjunctivitis
HAdV	human adenovirus
AIDS	acquired immunodeficiency syndrome
HIV	human immunodeficiency virus
A.P.C viruses	adenoidal-pharyngeal-conjunctival viruses
HVL	hypervariable loop
HVR	hypervariable region

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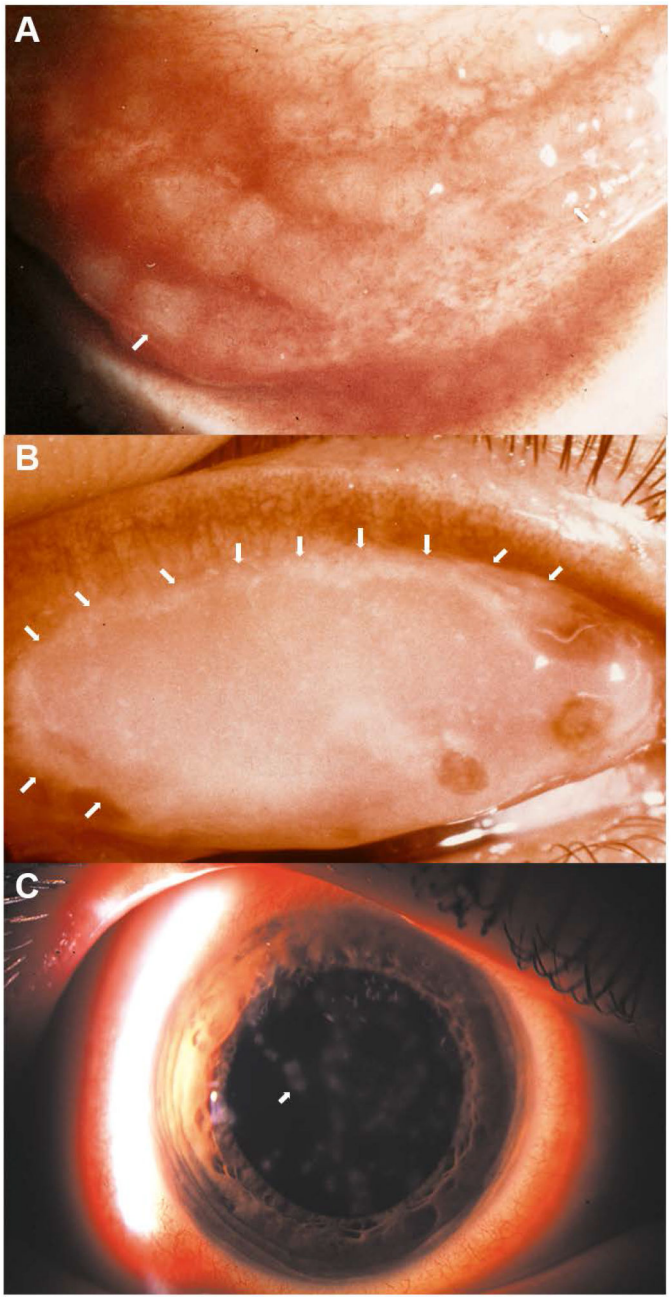

Fig. 1.

Photomicrographs of common clinical manifestations of ocular surface in epidemic keratoconjunctivitis (EKC). (**A**) Inferior conjunctival fornix of a patient with acute EKC shows conjunctival lymphoid hyperplasia presenting as jelly bean-shaped milky elevations of the mucosa (arrows point to two out of many follicles). (**B**) Superior eyelid tarsal surface with a conjunctival membrane (with visible margins of the membrane delineated by white arrows). (**C**) Corneal subepithelial infiltrates (arrow points to one of many such infiltrates). Image in C reproduced with permission.[264]

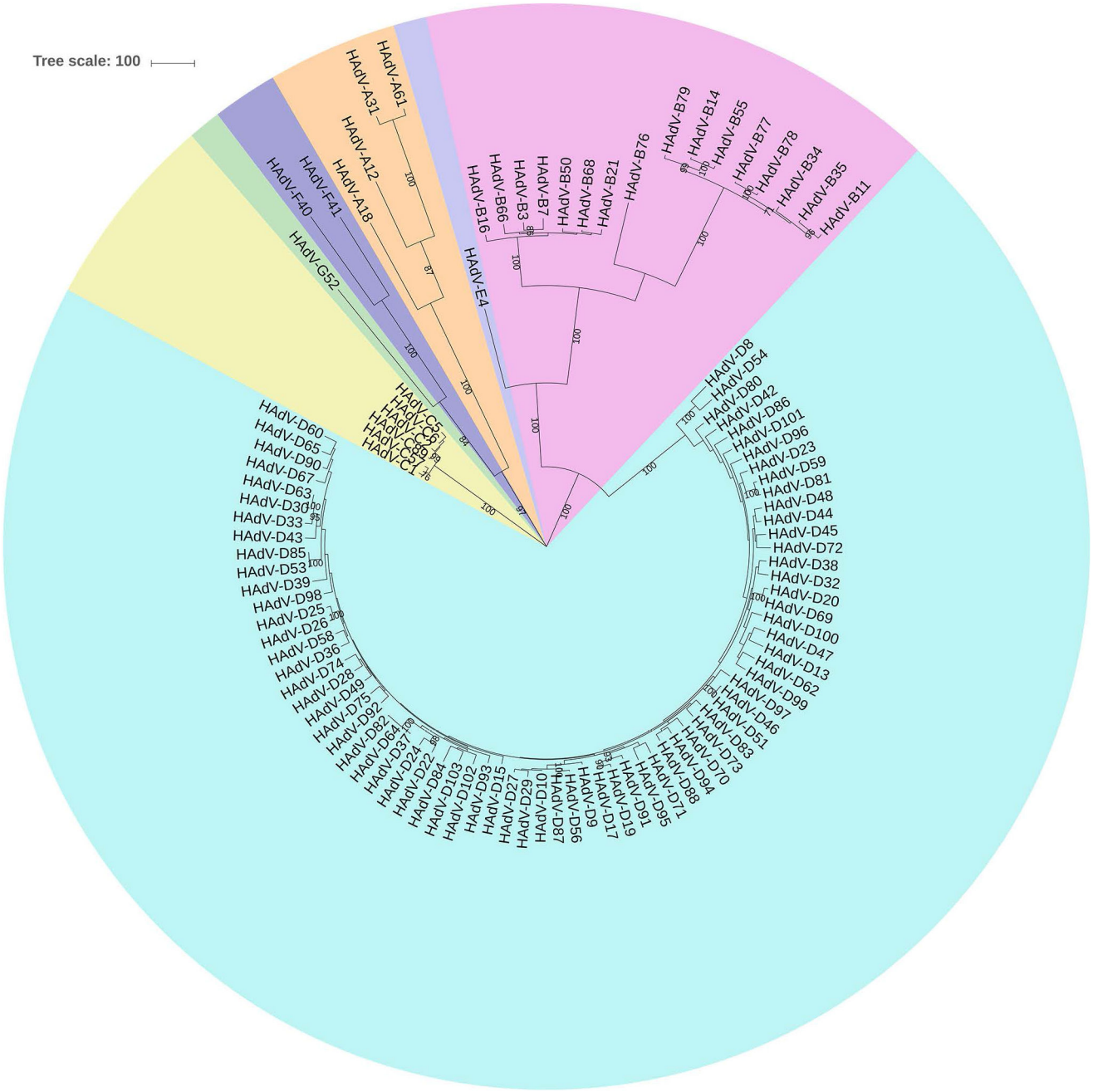

Fig. 2.

Phylogenetic analysis of human adenovirus (HAdV) DNA polymerase gene, including 103 genotypes, for HAdV species A-G. The tree was constructed in MEGA7 using the Maximum parsimony analysis with a bootstrap test of 500 replicates. HAdV-D is the largest species. The tree was obtained using the Tree-Bisection-Regrafting (TBR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The tree is drawn to scale with branch lengths calculated using the average pathway method and is in the units of the number of changes over the whole sequence. All

positions with less than 95% site coverage were eliminated and there were a total of 3270 positions in the final dataset.

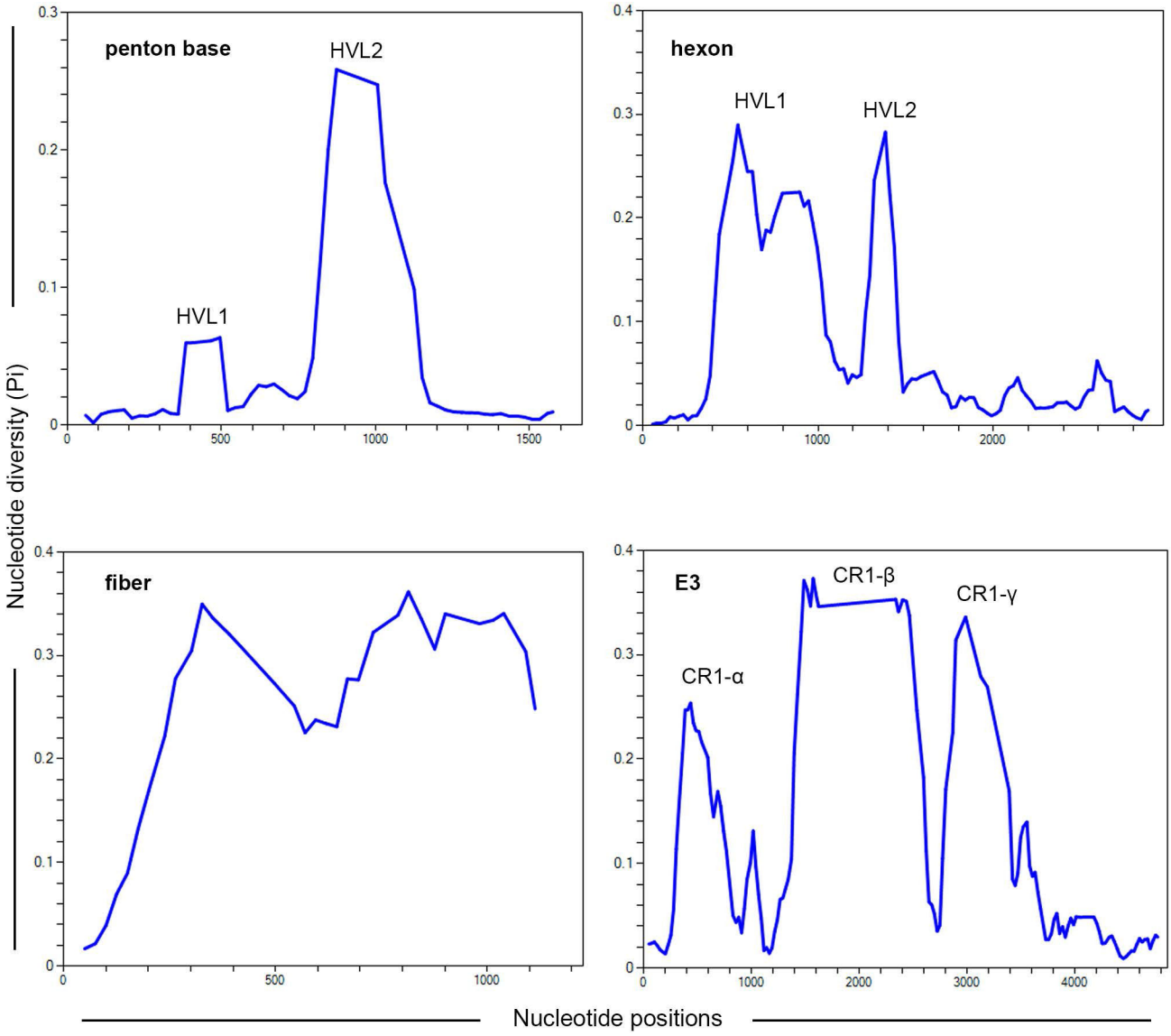

Fig. 3.

Nucleotide diversity Pi (π) plots showing the average number of nucleotide differences per site for the known hypervariable regions in HAdV-Ds, including penton base, hexon, fiber, and E3 CR1 genes, and performed with all 73 HAdV-D genotypes. The plot was constructed using DnaSP v6 with 100 nt window and 25 nt step size (gaps are excluded).

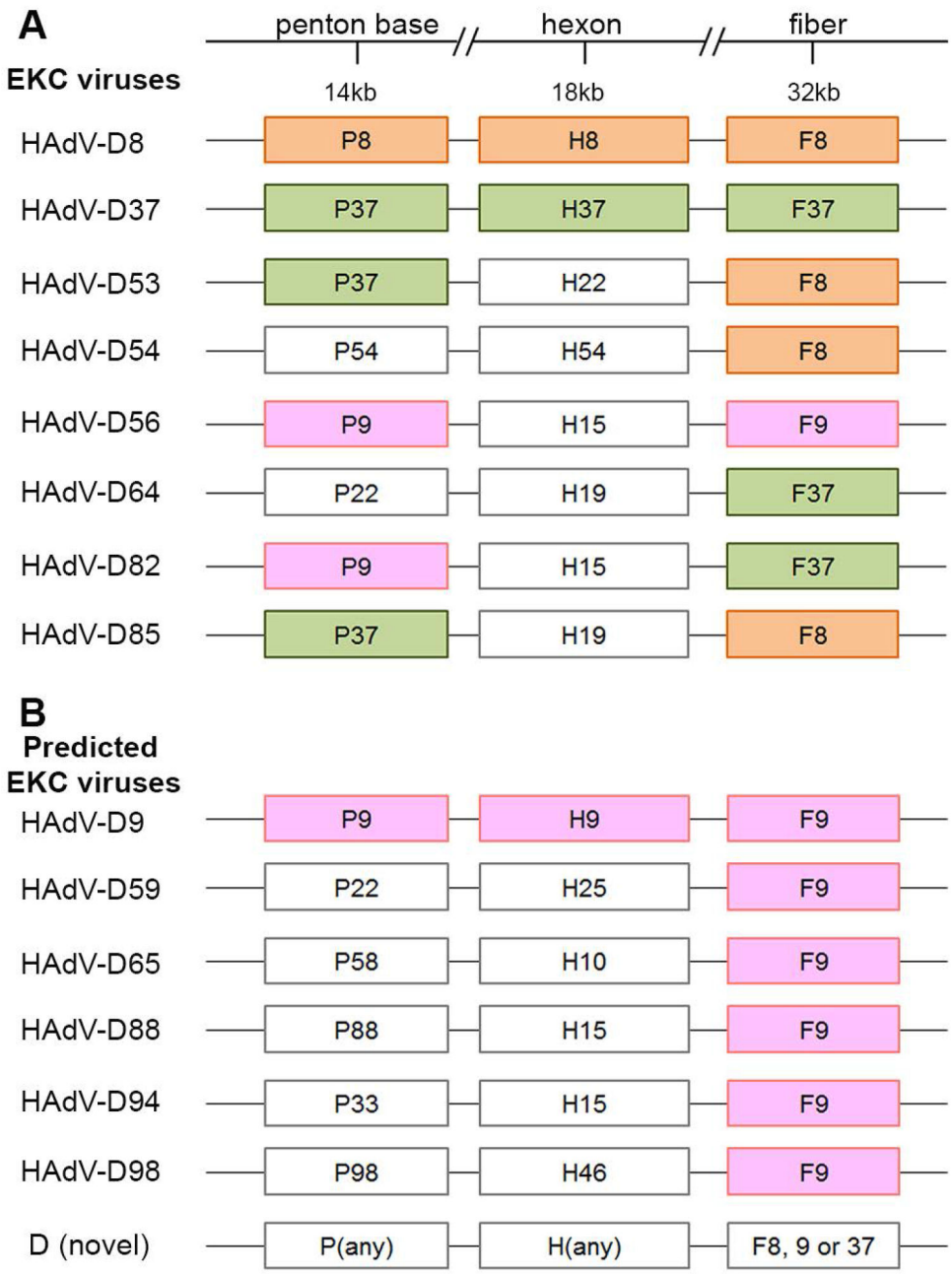

Fig. 4.

Molecular HAdV-D types known to cause epidemic keratoconjunctivitis (EKC) and those predicted to be possible causes. (**A**) The known EKC viruses all have a fiber gene from either type HAdV-D8, D9, or D37, with otherwise disparate hexon genes. (**B**) Putative EKC-causing viruses based on the aforementioned fiber gene association. It is proposed that any virus with the fiber knob of HAdV-D8, D9, or D37 is a potential cause of EKC.

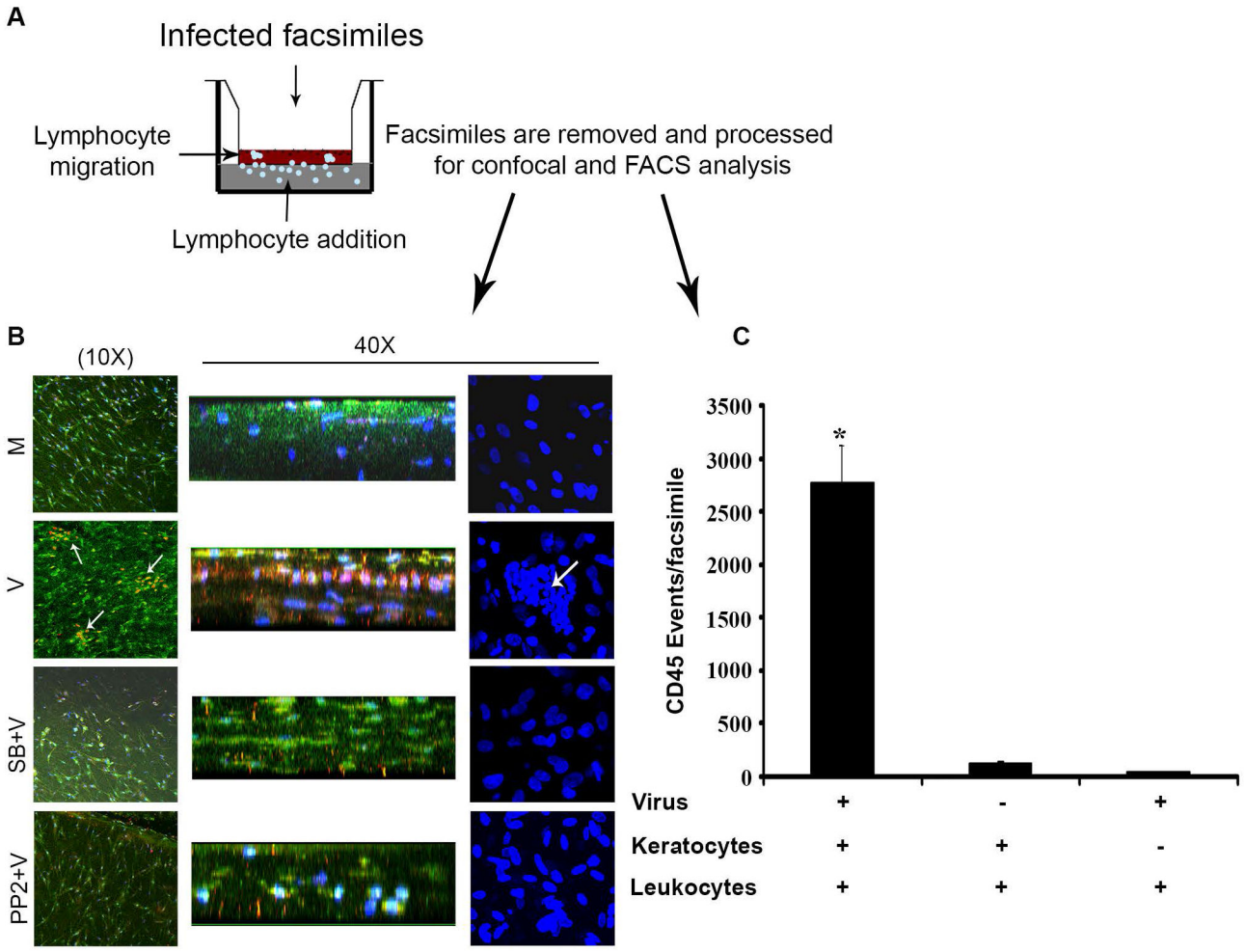

Fig. 5.

Cornea facsimile model of adenovirus keratitis. (**A**). Facsimiles shown in the sketch were generated in 12-ml transwell plates with a 3um pore size using primary human keratocytes mixed with collagen type 1, and layered with reduced growth factor containing Matrigel®. Infection with HAdV-D37 was performed overnight, and leukocytes then added to the lower chamber for one hour prior to harvest for confocal microscopy and flow cytometry for myeloperoxidase and CD45, respectively. (**B**) By myeloperoxidase staining (red/orange), as shown both *en face* (left column) and in cross section (stacked 3-D view, middle column), infected (V) corneal facsimiles developed neutrophil infiltration, whereas mock (M) infected facsimiles did not. DAPI staining (blue) to show cell nuclei (right column) showed similar pockets of cellular infiltration only in virus infected wells. Pretreatment with inhibitors of p38 (SB203580) and Src (PP2) blocked infiltration. (**C**) Flow cytometry for CD45 positive cells (leukocytes) in HAdV-D37 infected facsimiles showed CD45 events only in the presence of both virus and keratocytes. Therefore, virus alone was insufficient to induce chemotaxis. Figure modified with permission from Rajaiya et al.[215]

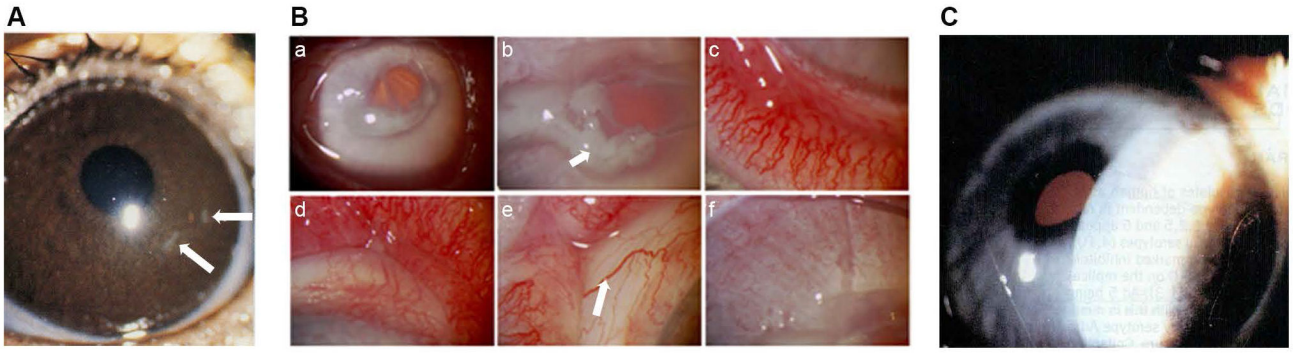

Fig. 6.

Cotton rat and rabbit models of adenovirus ocular infection. (**A**) The cotton rat model, figure adapted with permission from Tsai et al.[217] The image shown is of an adult cotton rat cornea 18 days after inoculation with HAdV-D8 (1×10^5 plaque-forming units). Arrows indicate subepithelial opacities observed two weeks after inoculation. (**B**). New Zealand White (NZW) rabbit ocular model, figure adapted with permission from Clement et al.[221] Image of Ad5-infected NZW rabbit eye at 7 days post-infection. **a**. Intact cornea. **b**. Discharge and exudates on the cornea (arrow). **c**. Lower eyelid inflammation/vascular dilation. **d**. Upper eyelid inflammation/vascular dilation. **e**. Prominent blood vessel (arrow) close to the caruncle of the eye. **f**. Corneal neovascularization. (**C**) Hollander rabbit corneal model, figure adapted with permission from Hauwere et al.[222] Rabbits were infected with HAdV-C5 by intracorneal and subconjunctival injections and topical instillation. The infected eye developed subepithelial opacities by day 56 post infection.

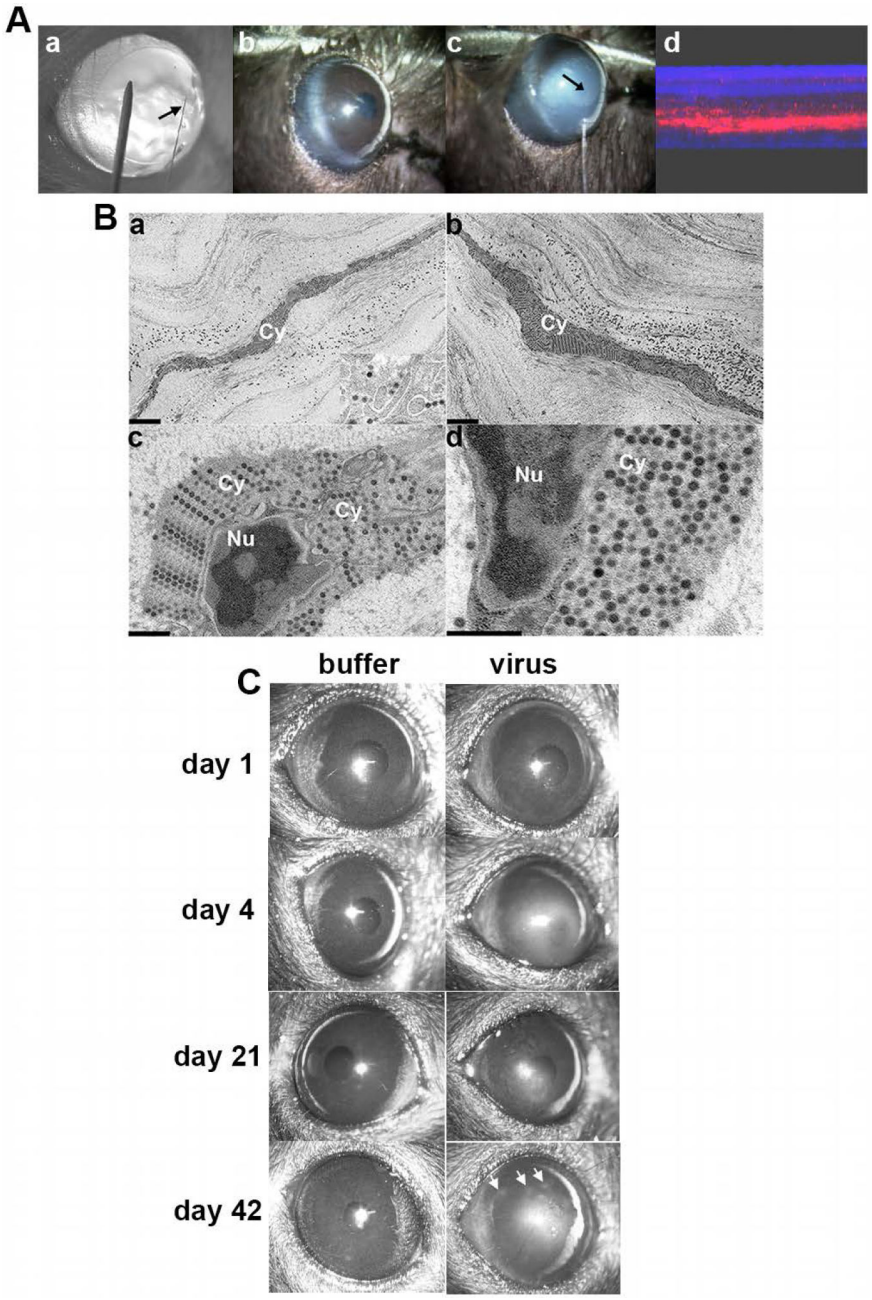

Fig. 7.

Mouse model of adenovirus keratitis. (**A**) Induction of the mouse model of adenovirus keratitis. **a**. BALB/c mouse cornea retroilluminated to show size of heat-pulled, glass micropipette needle (arrow) as compared to a 33 gauge metal needle. **b**. Diffuse illumination view of mouse cornea prior to, and **c.** during injection with HAdV-D37 or virus-free dialysis buffer using the glass needle (arrow) and a gas-powered microinjection system. The injection causes transient whitening of the corneal stroma (arrow points to tip of glass needle within the corneal stroma. **d**. Composite confocal image of a mouse cornea taken immediately after injection of Cy3 dye demonstrates successful intrastromal injection. (**B**)

Thin-section electron microscopy of C57Bl/6j mouse corneal stroma at intermediate time points after intrastromal injection of HAdV-D37. Micrographs show the corneal stroma at **a**, 4 hours, and **b-d**, 8 hours after injection. Intracellular structures are labeled as follows: Cy, cytoplasm; Nu, nucleus. The inset in **a** shows a higher magnification of intracellular virus. All micrographs show densely packed intracellular viral arrays. Scale bars: 2 μm in **a** and **b**, and 0.5 μm in **c** and **d**, and the inset in **a**. Adapted with permission from Mukherjee et al. [233] (**C**) Representative clinical photographs of C57BL/6j mouse corneas after mock infection with dialysis buffer or 10^5 TCID HAdV-D37 at days shown post infection. Buffer-injected corneas remained clear at all times. Opacities in HAdV-D37 injected corneas were seen as early as 1 day after infection, and appeared to peak at 4 days. The opacities then regressed slowly but in approximately one-third of mice recurred as characteristic subepithelial infiltrates at about 6 weeks after infection (day 42, right panel, arrows). Figure adapted with permission from Chintakuntlawar et al.[232]