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ADENOVIRUS VECTORS DELETED FOR GENES ESSENTIAL FOR VIRAL DNA REPLICATION

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1. ABSTRACT

Adenovirus (Ad) gene therapy vectors made replication defective by deletion of the E1 region (first-generation vectors) induce high-level inflammation that leads to loss of both transduced gene expression and transduced cells. First-generation vectors were initially considered to be incapable of viral DNA replication, but it is necessary to delete one or more of the genes, all in the E2 transcription unit, that encode proteins essential for Ad DNA replication to completely eliminate viral DNA replication. Vectors deleted for one or more of the E2 genes (second-generation vectors) induce reduced levels of inflammation in certain animal models and offer promise for understanding the mechanisms by which adenovirus vectors induce inflammation and how inflammation can be inhibited. While first-generation vectors dominated the initial human gene therapy trials using adenovirus vectors, second-generation vectors may offer greater promise and greater safety for clinical studies.

2. INTRODUCTION

Adenovirus (Ad) vectors, most of which are derived from Ad serotype 5 (Ad5), have become important transducing tools for studies *in vitro* and *in vivo* and have been used in a large number of gene therapy trials (http://www4.od.nih.gov/oba/rac/clinicaltrial.htm). While promising, first-generation Ad vectors suffer from the induction of strong innate inflammatory and adaptive immune responses that limit their usefulness (1-4). Knowledge of the host innate response to Ad vectors is progressing rapidly and adaptive immune responses to Ad vectors have been studied in detail. However, the mechanisms by which Ad vectors induce innate inflammatory processes, which exert a strong negative effect on first-generation vectors and a lesser, but still important, effect on helper-dependent vectors, are not well understood.

Ad vectors have been tested *in vivo* in a number of models, including human, chimpanzee, various monkeys, rats, cotton rats, and mice (including a number of different mouse strains). The substantial majority of studies have been undertaken in mice. A large number of organs have been targeted including liver, lung, skeletal muscle, cardiac muscle, and brain, with the substantial majority of assays targeting liver. The use of many animal

and organ models make it difficult to compare studies. However, general comparisons can be made. In designing and using Ad vectors, it is helpful to consider the biology of Ad in natural infections of humans.

3. BIOLOGY OF AD5

3.1. Natural Ad5 infections

Ad5 infections (reviewed in 5, 6) are common in childhood. The normal route of entry into the body is through infection of the pharyngeal epithelium. In young children, approximately 30-50% of infections are symptomatic, with sore throat and fever the most common symptoms. After infectious virus is produced in the pharyngeal epithelium, virus seeds the small intestine through a mechanism that is not clear. In the small intestine, the infection may be persistent, with fecal shedding of virus lasting days to more than one year. Infections of the small intestine appear to be asymptomatic. In severely immunocompromised patients, Ad5 infection can become systemic, and numerous tissues, including liver and lungs, may be infected (reviewed in 7-10). Systemic infections cause a high level of mortality.

Ad5 infection of liver cells *in vitro* and, in immunocompromised patients *in vivo*, is efficient. The efficiency of liver infection under appropriate conditions raises the question of why Ad5 does not normally infect the liver during the viremic phase of natural infections, an important point for Ad gene therapy vectors because transduction of mouse liver has been the most popular model for study.

The relatively low frequency of symptomatic infections of the pharyngeal epithelium and the apparent absence of symptoms during persistent intestinal infections by Ad5 suggest that the virus is not necessarily inflammatory in immunocompetent hosts.

4. AD VECTORS

4.1. First-generation vectors

Ad vectors are generally classed in three groups in a manner dependent on what Ad genes are deleted in the development of the vector. Most Ad vectors in use are derived from virus made replication-defective by deletion of the E1 region (first-generation vectors). The E1 region encodes E1A and E1B proteins, which have a variety of functions in the viral life cycle and are the primary transforming proteins of the virus (reviewed in 11-13). Deletion of the E1 region was initially thought to make the virus incapable of replicating its DNA. However, the effects of deletion of either E1A or E1B on viral replication *in vitro* can be overcome by infecting with a high multiplicity of virus (14) raising the possibility that, even at low multiplicities, viral DNA replication could occur in some cell types. More recently, it has been demonstrated that deletion of both E1A and E1B leads to a severe reduction in viral DNA replication *in vitro* that can also be overcome when cells are infected at high multiplicity (15, 16).

The question of whether Ad vector DNA replication occurs *in vivo* is important for understanding the mechanism(s) by which first- and second-generation Ad vectors differ in their inflammatory potential. However, it is a very difficult question to address, since low levels of replication will be obscured by the rapid loss of viral DNA that occurs after injection. The best evidence on this topic comes from Nelson and Kay, who used Ad vectors that had been grown in a cell line that methylates DNA at *Xho*I sites (17). Methylation at *Xho*I sites inhibits cleavage by *Xho*I. Replication of Ad DNA *in vivo* does not lead to methylation, so, assuming that complete methylation occurs during virus production, two rounds of replication will lead to production of unmethylated daughter molecules, restoring susceptibility to *Xho*I cleavage. After injection in a variety of mouse strains, viral DNA was not cleavable by *Xho*I, raising the possibility that viral DNA replication did not occur. As noted by the authors, a low level of viral DNA replication would have been difficult to detect. For example, a low level of replication in a small number of cells that limit the inflammatory reaction could occur and initiate an inflammatory reaction.

Most first-generation vectors are also deleted for the E3 region. E3 is not required for growth in tissue culture and its deletion provides additional room for insertion of foreign genes. E3 encodes a variety of proteins that act to inhibit innate and adaptive immune responses to the virus during productive infections (reviewed in 18-20). While the anti-inflammatory and immune inhibitory effects of E3 are of clear importance for the growth of the wild-type virus, the roles of E3 in protecting Ad vector-transduced cells from innate and adaptive immune responses are less clear. E3, or certain E3 proteins, have been found to be protective in certa*in situ*ations (21-23) but not in others (24, 25).

First-generation vectors are typically constructed and grown in HEK 293 cells (26), which express the E1 genes of Ad5 and complement E1-deleted vectors for high titer growth. These vectors are relatively easy to construct using recombination in a plasmid, a system originally developed *S. cerevisiae* (27) and then in *E. coli* (28-30). The recombinant vector chromosome is removed from the plasmid by restriction digestion and used to transfect complementing cells to generate virus. First-generation vectors grow to high titers and are relatively easy to purify.

4.2. Second-generation vectors

The second group of vectors, deleted for E4 or made replication incompetent by deletion of one or more of the E2 genes, is called second-generation vectors. Second-generation vectors require helper function, typically provided by a cell line that stably expresses the product(s) of the deleted Ad gene(s) for complementation of vector production and growth. Construction and screening of complementing cell lines can be time consuming. However, once an efficient complementing cell line is isolated, the second-generation vector can be prepared as for first-generation vectors using plasmid recombination in *E. coli* followed by transfection of complementing cells. Second-generation vectors typically grow to titers nearly as high as those of first-generation vectors, and purification of the two types of vector is quite similar. Second-generation

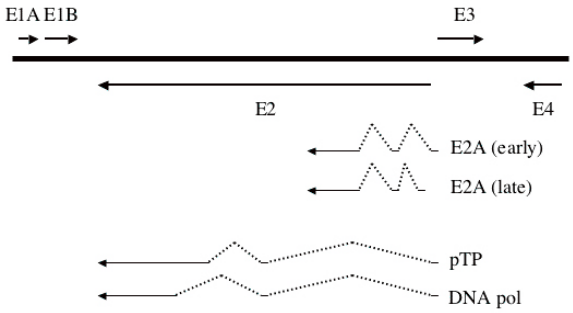

Figure 1. Schematic representation of the Ad early region transcription units. The Ad chromosome is represented by a thick horizontal line. Transcription units are indicated by arrows, with genes transcribed in the rightward direction above and in the leftward direction below the Ad chromosome. The splicing patterns of the E2 mRNAs are indicated below, with introns indicated by dashed lines. E2A expressioin is directed by two promoters, one active primarily during the early phase and one primarily active during the late phase of productive infection.

vectors induce reduced levels of inflammation relative to first-generation vectors in at least some circumstances and offer greater promise both as gene therapy agents and for understanding the mechanism(s) by which Ad vectors induce and inhibit inflammation. However, as discussed below, the greater potential of second-generation vectors has not led to their use in gene therapy trials.

4.3. Third-generation vectors

The third group of Ad vectors is deleted for all Ad genes and is variously called helper dependent, high capacity, gutless, or gutted. Growth of helper-dependent vectors typically requires a helper cell line, such as HEK 293, and a coinfecting helper virus to provide all of the packaging proteins and functions necessary for virus formation (31, 32). The coinfecting helper virus has a chromosome of different length such that the density of the helper and vector virus particles differ, permitting the vector to be purified using CsCl density gradient centrifugation. In addition, the helper virus usually has recombinase target sites flanking the cis-acting packaging element. The helper cell line expresses a recombinase that recognizes the target sites, and removes the cis-acting packaging element from the great majority of the helper virus chromosomes, thus increasing the ratio of vector:helper virus (32). Systems in which a second-generation, replication-incompetent helper virus is used are particularly promising for the production of helper-dependent vectors (33, 34). Helper-dependent vectors are more difficult to grow and purify than first- and second-generation Ad vectors. However, when efficiently purified from the helper virus, these vectors are considerably less inflammatory than are first-generation vectors, although they are inflammatory (35), and can induce severe complications, including death, when used at high multiplicity (36).

5. AD DNA REPLICATION

5.1. Mechanism of adenovirus DNA replication

Adenovirus DNA replication has been the subject of recent reviews (37, 38) and only a brief overview relevant to construction and use of vectors deleted for one or more of the genes essential for replication will be presented here.

The adenovirus chromosome is linear in the virion and does not form a circular intermediate at any time in the replicative cycle. The absence of a circular intermediate imposes specific constraints on the mechanism of DNA replication, including the requirement for the use of a priming mechanism that does not utilize RNA.

Inverted terminal repeats (ITRs) of 103 bp lie at the ends of the Ad5 chromosome and act as origins of replication. The 5' end of each strand in the infectious virion is covalently bound to a virally-encoded protein, called terminal protein (TP). TP is synthesized in a precursor form, preterminal protein (pTP), that acts as a primer for viral DNA replication. pTP is proteolytically processed to TP by the adenovirus encoded protease during maturation of virions. During the early phase of infection, newly synthesized pTP forms a high-affinity complex with newly synthesized adenovirus DNA polymerase (DNA pol) in the cytoplasm. The complex then translocates to the nucleus.

The pTP/DNA pol complex binds to the ITR through association of pTP with a highly conserved 11 bp sequence located at the 5' end of the ITR. This binding is facilitated by TP or pTP covalently associated TP with the 5'-most base in the ITR and interaction with host factors NF-1 and Oct-1. In the initiation complex, pTP is covalently bound to a dCMP residue, in a reaction presumably mediated by DNA pol, to a conserved Ser residue. This reaction appears to be completely conserved, as all adenoviruses examined to date share dCMP as the 5'-most nucleotide. Replication initiation also requires DBP, which appears to act through destabilization of the double helix to permit formation of the initiation complex. Because of the use of a protein-priming mechanism, one complete round of DNA synthesis leads to the formation of one double-stranded and one single-stranded Ad chromosome. DBP is synthesized in large amounts and binds to the displaced single stranded DNA. The single-stranded DNA may act as template for second strand synthesis through a mechanism involving hybridization of the ITRs. Alternatively, DBP could catalyze formation of double-stranded chromosomes from single strands of opposite polarity.

5.2. Genetics of Ad replication

The Ad-encoded proteins required for replication of Ad DNA are all encoded by the E2 region (Figure 1). DBP is encoded by the E2A region, with coding sequence located between 66.8 and 62.3 map units. pTP and DNA pol are encoded by the E2B region. The first three amino acids are common between the two proteins and are encoded by the 39.2-39.7 map unit exon. The main bodies of the coding regions lie between 29.4 and 23.8 map units for pTP and between 24.4 and 14.4 map units for DNA pol. Transcription of the E2 region is directed by two promoters, one primarily active during the early phase of expression that directs expression of all three E2 genes, and

one that is primarily active during the late phase and directs expression of DBP. E2 transcripts are differentially spliced and polyadenylated to yield the mRNAs encoding the three gene products. DBP is made in greatest abundance of the E2 proteins and is the major early Ad antigen in productive infections *in vitro*. pTP and DNA pol are synthesized in much smaller amounts.

Viruses temperature sensitive (ts) for specific functions offer powerful tools for studies of gene function as well as for screening of cell lines that stably express the wild-type gene. Temperature-sensitive mutations in the DBP and DNA pol genes were discovered in random screens of mutagenized Ad (39, 40). The widely used Ad5*ts*125 (DBP mutant) and Ad5*ts*149 (DNA pol mutant) exhibit tight temperature-sensitive behavior, with little Ad DNA replication at the non-permissive temperature of 39°C but efficient replication at the permissive temperature of 32°C. In contrast, Ad mutants ts for pTP function were not discovered in the random screens. However, ts pTP mutant viruses were constructed after two and four amino acid insertion mutagenesis (41-44).

One important consequence of the mechanism of replication is that DBP, pTP, and DNA pol are all essential for Ad DNA replication. A second important consequence arises from the fact that all of the adenovirus early region promoters are active in the absence of expression of the E1A transcriptional transactivator, albeit at reduced levels: no other adenovirus gene products are essential for Ad DNA replication (45-47). In a series of elegant experiments involving infection of HeLa cells with E1A and E1B deletion mutants, Shenk *et al.* demonstrated that high-multiplicity infections with viruses encoding either, but not both, the E1A or E1B genes led to efficient viral replication and formation of infectious virions that approached levels exhibited by the phenotypically wild-type virus (14). This work was not well known, and it was generally believed that deletion of the E1 region would make adenovirus incapable of replicating. More recently, the ability of viruses deleted for both the E1A and E1B genes to undergo viral DNA replication after high multiplicity infection of human tissue culture cell lines *in vitro* has been demonstrated (15, 16, 48).

6. NON-REPLICATIVE E2 PROTEIN FUNCTIONS

DBP and pTP have both been shown to have functions that are not directly involved in the mechanism of Ad DNA replication in addition to their direct roles in Ad DNA replication. These functions have been determined both through examination of the behavior of the isolated proteins in transfection assays and through examination of the effects of viral mutations on the infectious cycle. Examination of the literature did not indicate that Ad DNA pol has functions outside of replication of Ad DNA. It remains a possibility that such roles will be discovered, however. The non-replicative roles are important in considering the effects of deletion of the genes on the behavior of vectors. In particular, DBP has a number of functions not directly related to replication of Ad DNA that could affect gene therapy vector function.

6.1.Non-replicative functions of DBP

Perhaps the best characterized of the DBP activities not directly related to Ad DNA replication is its role in synthesis of Ad fiber. Fiber mRNA is encoded by the L5 region in the major late transcription unit and shares similarities in structure with other major late mRNAs. In particular, the 5' end of the mRNA consists of the tripartite leader, which facilitates translation late in infection after inactivation of the CAP binding protein. A fraction of the fiber mRNA differs from other mRNAs in that it also contains either the x- or y-leader. DBP localizes to both the nucleus and the cytoplasm. In the cytoplasm, DBP acts an RNA binding factor and facilitates translation of fiber mRNA (49-51). In monkey cells, translation of fiber mRNA is inefficient, but a specific mutation in the DBP coding sequence permits efficient translation and infectious virus formation (52, 53). This evidence suggests that DBP recruits a host factor that directs fiber mRNA translation.

DBP affects mRNA metabolism and transcription of certain adenovirus genes. DBP increases the rate of transcription of the major late transcription unit and decreases premature termination (54) ; acts as an autoregulator of its own production (55) ; alters the stability of certain mRNAs (56) ; and acts as a transcriptional activator of Ad genes during transient transfection (57). In addition to wild-type DBP, temperature-sensitive alleles of DBP exhibit altered behavior in RNA metabolism (58).

6.2. pTP function outside of priming replication

Non-replicative functions of pTP have not been studied in the detail to which DBP has, but pTP also has functions in addition to its role in priming Ad DNA replication. pTP (and TP) facilitate Ad gene transcription (59, 60), most likely through binding the Ad chromosome to the nuclear matrix (59-63). In addition, pTP expression has been reported to stabilize the Ad chromosome after transduction in vivo. Deletion of the pTP gene from a vector that encodes DNA pol and DBP led to instability of the chromosome after transduction of mouse liver (64). However, deletion of both the pTP and DNA pol genes did not lead to an increased rate of loss of the Ad vector chromosome after transduction in vivo (65). Helper-dependent vector chromosomes, which do not encode pTP, are maintained for extended periods of time, demonstrating that pTP expression is not generally required to maintain the vector chromosome in transduced cells. However, it remains a possibility that pTP prevents destabilization of the Ad chromosome in the presence of DNA pol expression.

Non-replicative functions of DBP, and to a lesser extent, pTP, raise the possibility that deletion of the DBP or pTP genes in Ad vectors may increase the safety of such vectors in that these deletions could lead to reduced expression of Ad genes and reduced effect of the vector on host cells relative to vectors encoding these genes.

7. CONSTRUCTION OF CELL LINES TO COMPLEMENT GROWTH OF AD VECTORS DELETED FOR E2 GENES

Production of replication-incompetent vectors derived by deletion of one or more of the genes essential for adenovirus DNA replication have generally relied on the construction of cell lines that express the gene(s) of

interest and complement growth of the deletion mutant virus. Since vectors are also usually deleted for the E1 genes, the cell lines must also express the E1A and E1B proteins. The E2 gene products, especially DBP, were originally considered to be toxic to cells, suggesting that successful development of complementing cell lines would require the use of tightly regulated, inducible promoters (66). However, numerous cell lines that constitutively express the E2 proteins at levels sufficient to permit efficient complementation for growth of viruses deleted in the corresponding gene have been developed (67-69), suggesting that the original problems were due to difficulties in screening for cell lines that expressed sufficiently high levels of the protein of interest to permit efficient complementation.

7.1.Considerations for the development of cell lines that complement E2 deletion mutant vector growth

Deletion of any of the three E2 coding regions leads to complete absence of viral DNA replication. Since there is no difference in replication potential, considerations about which gene(s) to delete in the construction of an Ad vector can be guided by the roles of the different proteins. One consideration in the development of a cell line that complements an E2 deletion mutant and a virus deleted for the same E2 gene arises from the non-replicative roles of the proteins. As discussed above, deletion of the DBP gene and to a lesser extent, the pTP gene may lead to increased safety because of potential reductions in the level of expression of Ad proteins and in effects on the host cell. Because DBP is expressed at higher levels than the other E2 proteins, deletion of the DBP gene may also lead to reduced immune recognition after transduction *in vivo*.

A second consideration arises from the compact nature of the Ad chromosome. Genes are tightly spaced and regions of DNA can contain important information transcribed from both DNA strands or overlapping regions can encode different proteins using alternative translation frames. The segment of DNA encoding DNA pol has a particularly large number of overlapping sequences (70). The 5' end of the main region encoding DNA pol overlaps the 3' end of the pTP coding sequence. The 3' end of the DNA pol coding sequence overlaps the IVa2 coding sequence and the bidirectional major late promoter. The DNA pol coding sequence also overlaps two of the three exons that make up the tripartite leader present in all of the mRNAs encoded in the major late transcription unit and the i-leader present primarily on L1 mRNAs. The pTP coding sequence overlaps the third segment of the tripartite leader. In contrast, the DBP coding region does not overlap other functions. The presence of sequences that are important for other functions embedded in the coding regions for DNA pol and pTP mean that deletions of the genes must be carefully constructed to avoid deleterious effects of inadvertent deletion of functions necessary for efficient virus growth.

Homologous recombination between the host copy of the E2 gene and the deleted E2 allele in the virus can lead to regeneration of viruses encoding the wild-type gene as is observed for the E1 genes (71). The presence of homologous sequences on both sides of the deletion facilitates recombination. The DNA pol main exon coding region has sequences at both the 5' and 3' ends that are required for expression of other genes, so simple deletions of the DNA pol gene alone require that the 5' and 3' ends of the gene be left intact, increasing the probability of recombination leading to reintroduction of the DNA pol gene into the vector. The 5' and 3' ends of the DBP gene do not overlap other required gene products, so it is relatively easy to construct complementing cell lines that do not have overlapping homologous sequence on either end of the gene. The main exon encoding pTP overlaps DNA pol at its 3' end, but there is no need to retain sequence at the 5' end, and it is relatively easy to develop cell lines that provide homologous sequence at only the 3' end of the pTP coding sequence. The frequency of recombination in pTP-expressing cell lines constructed using pTP coding sequence that overlaps at both the 5' and 3' ends the region deleted in a pTP-deletion mutant virus to reintroduce the pTP gene into the virus occurred at a relatively high frequency while the use of cell lines that express pTP from a construct that has overlapping homologous sequence at the 3' end only yielded an undetectable rate of recombination (Schaack *et al.*, unpublished). The presence of a significant population of replication-completent virus in stocks grown in a cell line that permits recombination mediated by homologous sequence on both sides of the deleted sequence raises concerns that there may be significant selective advantages for viruses that regain the E2 genes relative to E2 deletion mutants even in the presence of complementing expression of the deleted gene.

7.2. Screening for cell lines that express E2 gene products

Cell lines that express E2 proteins are usually constructed by stable transfection with plasmids containing the cDNA encoding the gene of interest. To screen selected cell lines, western or immunofluorescent analyses using antibodies that recognize the protein of interest can be used. In addition, the presence of the transfected gene and its mRNA can be examined. These processes are time consuming and limit the number of cell lines that can be tested. An alternative method is to take advantage of the temperature-sensitive Ads with mutations in the genes of interest. Isolated cell lines are incubated at the non-permissive temperature, typically 39° C, after infection with the temperature-sensitive virus. If a cell line expresses the protein of interest and complements the virus for growth at the non-permissive temperature, cytopathic effect becomes apparent. The use of viruses to screen cell lines increases the number of cell lines that can be tested. Unfortunately, this method of screening is negative, as the best cell lines support growth of the virus that kills the cells, so it is not as rapid as positive screening protocols. Temperature-sensitive viruses with mutations in the gene of interest have been used in screening for cell lines that express DBP, pTP, or DNA pol (67, 69, 72, 73).

7.3. Efficiency of complementation

Expression of the E2 proteins during productive infection is tightly regulated with respect to both the timing

and the level of synthesis. It is difficult to mimic the control of E2 protein expression observed during infection with the wild-type virus. In the absence of appropriate regulation, complementing protein expression may not lead to virus yields as great as those from growth of the wild-type virus. DNA replication of a pTP deletion mutant virus was reduced relative to that of a wild-type control in cells that expressed high levels of pTP (46). Further, there was not a strong correlation between the level of pTP expression and the efficiency of complementation for growth of a pTP deletion mutant virus (46 and Schaack *et al.*, unpublished data). In contrast, growth of DBP deletion mutant viruses was more efficient in cell lines that directed higher-level expression of DBP (34, 69).

In addition to potential problems associated with the control of E2 protein expression, the substitution of a foreign gene for the Ad E1 genes can lead to decreased virus yields, an effect that may be more pronounced with second-generation than first-generation Ad vectors (Schaack *et al.*, unpublished).

8. COMPARISON OF AD FIRST- AND SECOND-GENERATION VECTORS AS GENE THERAPY AGENTS

8.1. Ad vectors defective in E2A

In 1994, Wilson and colleagues published the first in a series of studies demonstrating that use of an Ad vector containing the ts allele of DBP from Ad5ts125 led to reduced induction of inflammation and prolonged expression of the transgene encoded by the vector compared to a first-generation vector (74-76). The increased efficacy of the DBP mutant vector could arise from DBP functions other than those directly involved in DNA replication. However, it is reasonable to assume that the reduced ability of the ts DBP to promote Ad DNA replication is responsible. The improved function of a vector with reduced potential to replicate its DNA raised the possibility that second-generation vectors deleted for on or more of the genes essential for replication would improve prospects for use of Ad vectors. However, other studies employing a vector with the same ts DBP allele did not demonstrate improved function (77). In addition, the use of a vector deleted for the E2A gene did not lead to reduced inflammatory responses relative to a first-generation vector (78). Interestingly, certain ts DBP mutants, including Ad5ts125, exhibit altered behavior in transformation assays (79). Other DBP ts mutants exhibit altered behavior in RNA metabolism (58). Gain-of-function effects of the ts DBP alleles could explain the different behavior reported by Wilson and colleagues relative to results obtained with vectors deleted for E2A but do not explain the differences reported by Fang et al. (77). Differences in cells targeted (76), in transgene encoded, or in animal or strain used in testing may all play roles in the differences observed in the effectiveness of the vectors mutated in E2A.

8.2. Ad vectors deleted for E2B genes

Ad vectors have been deleted for the pTP gene (80), the DNA pol gene (47), and both the pTP and DNA pol genes (33). Comparison of the effectiveness of the E2B deletion mutant viruses has demonstrated improved function relative to first-generation vectors in a variety of studies (80-83). In spite of the improved function of vectors deleted for E2B genes, strong inflammatory responses are still generated, in part due to adaptive immune responses to the foreign gene expressed at high levels (84). The use of a vector deleted for the DNA pol gene and encoding a transgene recognized as self led to improved function to a level similar to that of helper-dependent vectors (83), raising the possibility that with proper design, second-generation vectors may be as effective as helper-dependent vectors.

Recently, it was demonstrated that a replication-competent Ad that did not encode a transgene induced a modest but statistically significantly greater innate inflammatory response than a virus isogenic except for deletion of the pTP gene (85), suggesting that Ad vector DNA replication contributes to induction of inflammation. The mechanism by which replication competence of Ad vectors leads to induction of inflammatory processes is not clear. There is little if any DNA replication by first-generation vectors after transduction in vivo (17), so it appears that, for vector DNA replication to contribute, inflammatory responses would have to be exquisitely sensitive to vector DNA replication. It remains possible that first-generation Ad vector DNA replication occurs in only a small fraction of the transduced cells. The resulting modest increase in Ad gene copy number and Ad gene expression could contribute to inflammatory processes. However, this possibility remains to be tested.

8.3. Inflammation induced by Ad vectors

Ad vectors, especially first-generation vectors, are highly inflammatory in spite of the evidence that natural Ad infections may not be very inflammatory (5, 6). All Ad vectors induce innate immune functions shortly after vector administration (86-90) that can lead to death of the host (91). Adaptive immune responses develop within days of administration to both capsid proteins (1) and the transgene product (84). First- and second-generation Ad vectors induce stronger and more rapid adaptive immune responses, but foreign transgenes encoded by third-generation vectors also provoke adaptive immunity (92). Most comparisons of first-, second-, and third-generation Ad vector-induced inflammation have involved analyses beginning at least four days after transduction, at which time adaptive immune responses have begun. The presence of both innate and adaptive inflammatory responses complicates analyses of the nature of the responses.

Differences in innate responses induced by first- and second-generation Ad vectors are not well understood. In analyzing the increased potential of Ad second-generation vectors relative to first-generation vectors, it will be important to understand the roles each generation of vector plays in induction of both innate and adaptive immune responses.

9. USE OF AD VECTORS

9.1. Ad vectors for gene therapy

Ad vectors are in use in clinical trials both as corrective agents for gene replacement and as anti-tumor

agents (reviewed in 93). First-generation Ad vectors were the dominant type of Ad vectors used in trials of gene replacement therapy, with a limited number of trials involving the use of vectors with E4 deletions or the use of a vector carrying a ts E2A allele, but no trials utilized an Ad vector deleted for one of the E2 genes (http://www4.od.nih.gov/oba/rac/clinicaltrial.htm). The fact that human gene therapy trials employing Ad vectors were dominated by first-generation vectors in spite of the potential superiority of second-generation vectors may reflect the pressure to apply, rather than develop, gene therapy vectors. Since the death of a patient in trial using an Ad vector deleted for E1, E3, and E4 (94), enthusiasm for Ad vectors other than helper-dependent vectors for gene replacement therapy has declined dramatically.

9.2. Use of Ad transducing vectors for expression studies

The primary use of Ad vectors is in the transduction of cells in culture, where virtually all vectors in use are first-generation. Transduction with first-generation vectors used leads to the expression of the E2 genes and potentially to vector DNA replication (15, 16). In addition, the E4 proteins are expressed and affect a variety of cellular functions, including inhibition of p53 activity (95), regulating the activity of protein phosphatase 2A (96, 97), and altering the binding and activity of the transcription factor E2F (98-100). However, the effects of the Ad proteins have not presented severe limitations to the use of first-generation Ad vectors *in vitro*. In contrast, the effective use of first-generation Ad vectors to deliver a gene *in vivo*, typically to mice, is significantly limited by the inflammatory response induced and the resultant reduced duration of transgene expression. Second-generation Ad vectors offer potential improvement of function for such studies *in vivo*.

10. SUMMARY

Second-generation Ad vectors show greater potential as gene therapy agents in at least some circumstances than do the much more commonly used first-generation vectors. Recent efforts to understand the contributions of Ad early region proteins to the induction and inhibition of innate inflammatory responses (85) raise the possibility that second-generation vectors can be modified to make them as effective as helper-dependent vectors for use as gene therapy agents. The relative ease with which they can be produced offers a significant advantage and support for continuing efforts to understand and develop effective second-generation Ad vectors.

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