Diversity of adenoviruses in humans and in non-human primates
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Chapter 13

General Discussion
One of the reasons the promise of adenoviral vectors has been difficult to realize is the high efficiency with which antibodies against the capsid proteins are able to neutralize the infectivity of the virus. Based on kinetic studies it was calculated that as few as one antibody molecule directed against the hexon may be sufficient to prevent adenoviral infection (1). Those subjects in whom anti-vector titers are low enough so as not to prevent transduction, the first exposure to an adenovirus vector usually precludes efficacious re-administration. However the great number of available serotypes provides an opportunity to utilize this natural biological diversity for the construction of a library of adenoviral vectors based on criteria such as low seroprevalence in target populations, ease of manufacture of high-titer stocks, and in case of vaccines, good elicitation of humoral and cellular immunity against the encoded transgene.

The original adenoviral vectors were generally based on HAdV-5 principally because of the fact that it is easy to grow to high titers in the laboratory which led to it being used as a model organism, which in turn has led to an accumulated body of knowledge and reagents. Unfortunately infections with HAdV-5 and related strains are common in childhood and thus many prospective subjects may not be able to benefit from such a vector. Adenoviruses obtained from non-human sources offer the possibility that they would escape neutralization in humans who are unlikely to have “seen” the virus previously. The work described in this thesis describes the development of new adenovirus serotypes isolated from non-human primates, principally chimpanzees, for possible use as vectors. An important consideration was that the non-replicative vector created by deletion of the E1 genes should be adequately complemented in cell lines such as HEK 293 or PER.C6 that are already approved by regulatory authorities for the manufacture of adenoviral vectors to be used in humans.

Initially four chimpanzee adenoviruses that had been isolated from chimpanzees in the 1960s (2, 3) were candidates for vector construction. These adenoviruses belong to species E for which only one human adenovirus serotype, HAdV-4, has been defined. It had been shown that one of these chimpanzee adenoviruses, SAdV-25 (also called C68), could be deleted in E1 and this deletion could be complemented in HEK 293 cells (4). Based on this success, the three other species E chimpanzee adenoviruses, SAdV-22 (Pan 5), SAdV-23 (Pan 6) and SAdV-24 (Pan 7), as well as an unrelated species B chimpanzee adenovirus – SAdV-21 (C1/Bertha) (5), were fully sequenced. The analyses of the sequences are described in chapter 4. The sequence information was used to devise cloning strategies whereby the complete genomes of the E1-deleted adenoviruses could be cloned into a single plasmid (chapter 6). The creation of these plasmid molecular clones allowed the facile insertion of transgene cassettes from a shuttle vector into the cloned adenovirus genome. A single E.coli colony could be picked and the plasmid DNA prepared from it could be used to transfect the E1-complementing cell line HEK 293 cells to rescue clonally pure adenoviral vectors.

The quality of the T cell response elicited by one such chimpanzee adenovirus vector based on SAdV-24 was tested in mice in an influenza challenge model (chapter 12). Either SAdV-24 or HAdV-5 vectors were used to immunize mice against the nucleoprotein of an H1 strain (A/PR8/34) of influenza. The vaccinated
mice were lethally challenged by homologous (mouse-adapted A/PR8/34) and heterologous influenza strains (two highly pathogenic H5N1 strains). Protection against homologous challenge was good and a significant survival benefit was observed in one of the heterologous challenges.

Cross-neutralization experiments showed that SAdV-22, -24 and -25 belonged to the same serotype and SAdV-23 belonged to a different serotype (chapter 6). Thus SAdV-22, -24 and -25 could not be used as different vectors or in prime-boost combination regimens. Interestingly a three-dimensional computer reconstruction of the hexon trimer structures (John Rux, Wistar Institute, Philadelphia, personal communication) of SAdV-22, -23, and -24, compared to SAdV-25 showed that the outwardly disposed hypervariable loops accounted for the differences between SAdV-23 hexon with that of the other isolates (Figure 1). Re-administration experiments were performed in mice where mice were administered alpha-1 anti-trypsin (A1AT) encoding vectors (based on SAdV-22, -23, -24, and -25) after having been previously

Figure 1. Molecular modeling of chimpanzee adenovirus hexons. Ribbon representations of the SAdV-22, SAdV-23, and SAdV-24 hexon homology model structures showing the trimer viewed from the top, i.e., the exterior surface of the virion (upper row), and trimer viewed from the side (lower row). The structures are colored to indicate the sequence differences between the indicated hexon and SAdV-25 (Ad C68). The colors range from blue (conserved) to yellow (conservative) to red (non-conserved). The sequence divergence between SAdV-23 and SAdV-25 is much greater than that for either SAdV-22 or SAdV-24. This variation is seen to be primarily localized to the external loops at the top of the molecule and corresponds to the positions of the type-specific epitopes.
given one of the other serotypes. Measurement of serum A1AT levels confirmed the predictions based on the sequence and serology data that SAdV-22, -24, and -25 could also be considered as a single serotype with respect to the efficacy of re-administration and that SAdV-23 was sufficiently different so that re-administration was feasible.

Sequencing of the species E chimpanzee adenoviruses SAdV-23 and SAdV-24 (originally called Pan 6 and Pan 7 respectively) had shown that the sequence identity between the two viruses across the length of the genome was very high. However as discussed above, the two viruses are serologically distinct, i.e., high-titer neutralizing anti-sera raised against either virus only poorly neutralized the other virus. In conformity with this, mice that had been administered either SAdV-23 or SAdV-24 could be re-administered with the other vector. Because the main sequence differences between SAdV-23 and SAdV-24 were in the hexon hypervariable loops (as illustrated in Figure 1) and in the fiber knob (the penton base proteins are identical), it was of interest to determine the relative importance of the hexon and the fiber knob towards contributing neutralization epitopes. This was tested by constructing chimeric adenoviruses where the hexon and fiber proteins were exchanged (chapter 10). The efficiency with which these chimeric adenoviruses could be neutralized by high titer anti-SAdV-23 and anti-SAdV-24 antisera was determined. Re-administration experiments were also carried out. The data indicated that both hexon and fiber contributed neutralization epitopes and that anti-hexon antibodies were more potent in neutralization as well as preventing re-administration in vivo.

The sequencing of SAdV-21 (chapter 4) had shown that it belonged to species B unlike the other four chimpanzee adenoviruses that were available at the time. However species B adenoviruses cannot be complemented in cell lines such as HEK 293 that expresses the HAdV-5 E1 genes in trans. To overcome this problem in the construction of a SAdV-21-based E1-deleted vector, a method was devised to enable species B adenoviruses to be complemented in such cells. This involved the construction of a chimeric vector where only the central portion of the genome that encoded the principal capsid components, including the penton base, hexon, and fiber, belonged to the species B genome, and the flanking sections belonged to a species E or a species C adenovirus. This methodology is described in chapter 7. Such a chimeric vector – where the central portion of the genome belonged to SAdV-21 (species B) and the flanking sections belonged to SAdV-22 (species E) – was tested in rhesus macaques and mice (chapter 11). The vaccinated animals were shown to mount antibody and T cell responses against the encoded transgene (Ebola glycoprotein). The mice were found to be protected against a lethal Ebola challenge.

The success achieved in the construction of vectors based on chimpanzee adenoviruses was an incentive to test whether adenoviruses that had been isolated from monkeys would also be suitable for vector construction especially because many such adenoviruses were publicly available. However of the several monkey adenoviruses tested, it was found that only SAdV-7 could be both grown to high titer in human cell lines and be complemented for E1 in HEK 293 cells (data not shown). SAdV-7 had been isolated in
the 1950s from primary monkey kidney cells being used to grow polio virus (6). The complete sequence of SAdV-7 was determined (as well as the complete sequences of three other monkey adenoviruses, SAdV-6, SAdV-18, and SAdV-20, chapter 5) and vector construction by plasmid molecular cloning was carried out (chapter 9). Interestingly, sequencing of SAdV-7 showed that the virus isolate that had been propagated had an extensive deletion in the E1 region such that the E1a gene product was severely truncated (51 residues instead of the ~260 residues present in the closest adenovirus homologues SAdV-1 and HAdV-52). Because of the cell immortalization property and tumorigenic potential of the E1a protein, there is an extensive body of knowledge with respect to the various domains of the E1a protein and the regions that are necessary or dispensable for adenoviral growth, as reviewed (7-9). There is no precedent for such a short E1a protein being adequate for viral replication. The mechanism by which this SAdV-7 isolate can replicate is not clear. Both replication-deficient as well as replication-competent vectors could be constructed using SAdV-7 (chapter 9).

Because of the experience with non-human primate adenoviruses, where adequate growth and complementation in HEK 293 cells could be achieved with chimpanzee adenoviruses, but with only limited success using monkey adenoviruses, it was decided to try and expand the repertoire of available adenoviruses from chimpanzees in particular and apes in general. Based on a report in the literature that chimpanzee adenoviruses could be cultured from stools (10), chimpanzee, bonobo, gorilla and orangutan stool samples were obtained from primate facilities and zoos in the United States. Adenoviruses present in the stool samples were recovered by applying stool filtrates to cultures of the human lung carcinoma cell line A549 that is commonly used to propagate adenoviruses. Adenoviruses could be cultured at a high frequency from all samples except from orangutans (even though these samples were also positive for adenoviral DNA by PCR). Thirty of the ape adenoviral isolates were completely sequenced as described in chapter 3. As a result it was possible to do a phylogenetic analysis of the ape adenoviruses and compare them to available human adenovirus sequences. It was observed that in contrast to monkey adenoviruses and other non-human adenoviruses that form their own distinct clades, the ape adenoviruses that had been isolated could be classified into the existing human adenovirus species. All the new isolates were seen to belong to HAdV-B, HAdV-C or HAdV-E species. Bioinformatic analyses indicated that this was possibly due to cross-species transmission events that had occurred between humans and apes over the approximately 10 million years that apes and humans have diverged from their common ancestor. Clear evidence for recombination between adenoviruses was also obtained.

Subsequent to the isolation and sequencing of novel ape adenoviruses, twenty of the isolated adenoviruses were molecularly cloned and E1-deleted constructs were created as described in chapter 8. In case of species B isolates, the methodology described in chapter 7 for creating chimeric clones was used. The E1-deleted clones of plasmid molecular clones were transfected into HEK 293 cells to rescue recombinant adenoviral vectors.
The observation that adenoviruses could be easily cultured from asymptomatic ape stools and subsequently also from stools collected in the wild (11) is in contrast to the fact that live adenoviruses can be cultured from normal human stools in only 2 to 3% of cases (12, 13). However, it was of interest to determine whether the presence of adenoviruses in human stools was not a true surrogate for the presence of adenoviruses in the human gut, i.e., whether adenoviruses were in fact present in the human gut at a higher frequency. Because other groups had shown that adenoviruses can set up persistent infection in lymphoid cells (14-16), it was decided to test human gut tissue as well as lymphocytes isolated from human gut tissue for the presence of adenoviral DNA by PCR of a conserved region of the adenoviral DNA polymerase gene. (chapter 2). Both CD4+ and CD8+ fractions of human gut lymphocytes were found to harbor adenoviral DNA at a high frequency. Additionally, there was a wide diversity of adenoviral sequences that could be isolated, including multiple sequences from the same individual. Moreover, although adenoviruses could not be recovered from lymphocyte cultures, the presence of adenoviral capsid (hexon) gene transcripts could be demonstrated in most samples. A previously unknown exon was identified in some species B adenovirus spliced major late transcripts.

As was seen with apes, macaques were also found to shed adenoviruses but similarly to human samples, culture of stools obtained from asymptomatic rhesus macaques from various primate facilities on monkey cell lines yielded adenoviruses in only 1 – 16% of samples. Nine of these newly isolated rhesus macaque adenoviruses were completely sequenced (chapter 5). Analyses of the genomes indicated that most of these isolates clustered into a distinct new clade (proposed to be called SAdV-B), which like SAdV-A and HAV-F contains two fiber genes. However SAdV-B has the distinct feature that the second fiber gene, which carries the CAR (cellular receptor) - binding knob, has a shorter shaft domain than the first fiber gene. A cellular receptor that can bind to the first fiber knob has not been identified to date, but the fact that this knob is present on the longer fiber shaft in the newly isolated macaque adenoviruses indicates that it is probably important in the tropism of these adenoviruses.

Adenoviruses are common pathogens that most of the human population is exposed to at an early age. In some cases this exposure results in a coryza-like illness, although more severe forms of upper respiratory tract disease can occur in situations like military boot camps. The adenoviral species mostly responsible for these outbreaks are HAdV-B, HAdV-C and HAdV-E. Other adenoviral species are associated with pathologies such as diarrhea and keratitis (17). However the natural history of adenovirus infections following the resolution of the acute episodes remains unclear. Adenoids and tonsils are known to harbor adenoviruses and the work described in this thesis extends this observation to lymphocytes that are present in the gut-associated lymphoid tissues in the lower part of the gastro-intestinal tract. Macaques and apes shed adenoviruses in their stools and similarly, the human gut also appears to be colonized by adenoviruses. In this thesis, the practicality of using the adenoviruses that colonize the gastro-intestinal tract of apes and macaques to create gene transfer vectors has also been explored. The use of such vectors may allow for their use in human populations where natural immunity may preclude the efficacious use of adenoviral vectors based on human adenovirus serotypes.
REFERENCES