Update on adenovirus and its vectors

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Introduction

Adenoviruses have been characterized extensively since their initial description in the early 1950s (Hillemann & Werner, 1954; Rowe et al., 1953) and there is now a panoply of observations on the properties of many of the virus gene products. Nevertheless, there is still a lack of understanding of a number of the molecular mechanisms that operate in the infected cell, particularly in respect of how the virus gene products interact with cellular components and of the nature of the responses mounted by the host in response to infection.

In this regard, it is significant that, although there were almost 4000 references to adenoviruses in the 3 years from 1997 to 1999, most of these have been concerned with the results of investigations using adenoviruses as vectors and relatively few have dealt with the basic virology and immunology of virus infection. Indeed, it is now accepted that the initial enthusiasm for utilizing adenovirus gene vectors in therapy was rather prematurely optimistic and was perhaps over-hyped. In its place, there is a realization that targeting the vector effectively is not so straightforward and that, more importantly, the efficacy of host defences has not been fully appreciated and must be adequately addressed.

This paper reviews the current knowledge in the field of adenovirus vectors as well as advances in our understanding of the properties of the adenovirus gene products. Particular emphasis has been made on developments over the last two to three years. There have been a number of reviews examining different aspects of the vector field, and the reader is referred to these (Benhououd et al., 1999; Hitt et al., 1997; Zhang, 1999) for more comprehensive coverage. The expectation remains that a better understanding of the total spectrum of the virus–cell and virus–host interactions will lead to the design of vectors that provide more efficient delivery along with minimal deleterious host reactions.

General properties of adenoviruses

Adenoviruses have a characteristic morphology (Stewart et al., 1993), with an icosahedral capsid consisting of three major proteins, hexon (II), penton base (III) and a knobbed fibre (IV), along with a number of other minor proteins, VI, VIII, IX, IIIa and IVa2 (Fig. 1). The virus genome is a linear, double-stranded DNA with a terminal protein (TP) attached covalently to the 5′ termini (Rekosh et al., 1977), which have inverted terminal repeats (ITRs). The virus DNA is intimately associated with the highly basic protein VII and a small peptide termed mu (Anderson et al., 1989). Another protein, V, is packaged with this DNA–protein complex and appears to provide a structural link to the capsid via protein VI (Matthews & Russell, 1995). The virus also contains a virus-encoded protease (Pr) (Weber, 1976; Webster et al., 1989), which is necessary for processing of some of the structural proteins to produce mature infectious virus.

Members of the adenovirus family (Adenoviridae) infect a great variety of post-mitotic cells, even those associated with highly differentiated tissues such as skeletal muscle, lung, brain and heart. Since they deliver their genome to the nucleus and can replicate with high efficiency, they are prime candidates for the expression and delivery of therapeutic genes. They have a wide host-range and are currently divided into three genera with further subdivision into species (also termed subgenera or subgroups) A to F. Division of human serotypes, based mainly on immunological criteria, has historically been the basis of classification (Benkő et al., 1999; Lukashok & Horwitz, 1998; Mautner, 1989). Some adenoviruses produce tumours in animals and can transform cells in vitro, but no reference to these characteristics will be made here.

Early events in adenovirus infection

The adenovirus infectious cycle can be clearly defined into two phases. The first or ‘early’ phase covers the entry of the virus into the host cell and the passage of the virus genome to the nucleus, followed by the selective transcription and translation of the early genes. These early events modulate the functions of the cell so as to facilitate the replication of the virus DNA and the resultant transcription and translation of the late genes. This leads to the assembly in the nucleus of the structural proteins and the maturation of infectious virus. The early phase in a permissive cell can take about 6–8 h (depending on a number of extraneous factors), while the late phase is normally much more rapid, yielding virus in another 4–6 h.

The adsorption of virus to target cell receptors involves high-affinity binding to cell receptors via the knob portion of
Fig. 1. Structure of adenovirus. The locations of the capsid and cement components are reasonably well defined. In contrast, the disposition of the core components and the virus DNA is largely conjectural.

The fibre; for a review see Chroboczek et al. (1995). The prime receptor for the human subgroup C adenoviruses was shown to be identical to that for coxsackie B virus (Bergelson et al., 1997) and has therefore been termed the coxsackie/adenovirus receptor (CAR). This has subsequently been shown to be a plasma membrane protein of 46 kDa belonging to the immunoglobulin superfamily and to contain extracellular, transmembrane and cytoplasmic domains (Tomko et al., 1997), with the extracellular domain being sufficient for attachment (Wang & Bergelson, 1999). A more recent study has indicated that the adenovirus CAR does not completely overlap the coxsackievirus receptor (Tomko et al., 2000). A comprehensive survey of representative members of all the human adenovirus species A to F (Roelvink et al., 1998) suggested that they all
bound to CAR with the exception of members of the subgroup B, which appear to recognize a different receptor (Stevenson et al., 1995). In the same study, it was also noted that adenovirus serotype 41 (Ad41) (of subgroup F) has two fibres of different lengths, and only one of them binds to CAR. Since Ad41 readily infects cells of the gastrointestinal tract, it seems likely that the other fibre will adsorb to a different cell receptor, perhaps displayed on enterocytes. Some cells, such as those of haemopoietic origin, appear to be largely refractory to productive infection by human adenoviruses 2 and 5 and do not display CAR molecules on their plasma membranes (Mentel et al., 1997). This suggests that receptor recognition could be one of the key factors involved in cell tropism. In an attempt to modify cell tropism, fibreless adenoviruses have been constructed. Not surprisingly, these particles showed drastically reduced infectivities and were extremely unstable. The low level of infectivity that could be detected possibly operated in some cell systems (Huang et al., 1999; see below). Recent investigations have succeeded in defining the receptor-binding motif on the three-dimensional structure of the fibre head (Kirby et al., 1999; Santis et al., 1999) as well as on the CAR (Kirby et al., 2000). It has also been shown that receptor recognition can be altered by switching fibre heads from other subgroups (Miyazawa et al., 1999). Experiments have also been carried out that have substituted or added other receptor-binding motifs (Hidaka et al., 1999) in the fibre knob; see below. It is interesting to note that some adenovirus serotypes seem to have additional specificities of binding, suggesting that the CAR receptor may be part of a family of receptors (Segerman et al., 2000). Indeed, another receptor, the histocompatibility class I molecule, also a member of the immunoglobulin superfamily, has been shown (Hong et al., 1997) to be available for the subgroup C viruses. Moreover, a recent observation suggests that Ad37, a member of subgroup D, appears to bind to sialoglycoprotein receptors (Arnberg et al., 2000), indicating that receptor specificities are wider than was at first thought.

After the initial interaction of the virus with the fibre receptor, entry of the virus proceeds via clathrin-mediated endocytosis. The critical recognition mechanism for this process is an RGD motif that is exposed on the penton base (Stewart et al., 1997) and interacts with cellular αvβ3 integrins (Wickham et al., 1993). There appears to be direct binding of the virus penton base to the integrins in the presence of divalent cations (Mathias et al., 1998). Integrins normally react with the extracellular matrix to facilitate adhesion, differentiation and other cell–cell phenomena (Meredith et al., 1996). They form a large family of heterodimeric receptors and it appears that integrins αvβ3 and αvβ5 both support adenovirus internalization. It is noteworthy that integrin αvβ5 is expressed on human bronchial epithelial cells, a major site of primary adenovirus infection in vivo (Mette et al., 1993). Integrins may also play an important part in defining tropism in some situations such as the intestinal epithelium (Croyle et al., 1998c) while in others, such as hepatocytes, they have a minimal role (Hautala et al., 1998).

Interaction of the virus with the plasma membrane can induce a number of signalling pathways and there is good evidence for the activation of the phosphoinositide-3-OH kinase (PI-3K) pathway, which in turn triggers the Rho family of GTPases and the polymerization and reorganization of actin to facilitate endocytosis (Li et al., 1998; Rauna et al., 1999). As early as 20 min post-infection, activation of the Raf/mitogen-activated protein kinase (MAPK) pathway and consequential production of IL-8 have been observed (Bruder & Kovessi, 1997). Since the activation of Raf/MAPK is insensitive to the addition of cycloheximide and is sensitive to prior heating of the virus inoculum at 56 °C, it seems plausible that the initial events at the cell membrane are triggered by a structural component, and this could be via the penton base, since it is heat sensitive (Russell et al., 1967). Triggering these pathways may act as an ‘early-warning system’ for the induction of defence mechanisms induced in the host (see below).

As noted above, progress of the virus through the endosomes and into the cell cytoplasm is normally mediated by clathrin and the coated pit pathway (Wang et al., 1998). Thereafter, the virus-encoded protease appears to assist in the further disruption of the virus capsid by the proteolysis of the structural protein VI (Greber et al., 1996), which functions as a linker between the capsid and the core components (Matthews & Russell, 1994, 1995). The partially disrupted virus is then transported to the nuclear membrane and the genome is passed through the nuclear pore and into the nucleus, where the primary transcription events are initiated. The passage through the cytoplasm to the nucleus has been postulated to be mediated by the association of the virus core (the virus DNA and the covalently attached TP, together with the basic proteins VII and V and mu peptide) with a cellular protein, p32 (Matthews & Russell, 1998b). The p32 protein is primarily located in the mitochondria but can also be detected in the nucleus, and it has been suggested that it is a component of a cellular transport system that shuttles between the mitochondria and the nucleus and that the virus can hijack this system to gain access to the nucleus. This passage to the nucleus is relatively rapid and also involves the participation of dynein and microtubules (Leopold et al., 1999; Suomalainen et al., 1999). Virus-like particles can be detected at the nuclear membrane by electron microscopy within 1 h of infection (Dales & Chardonnnet, 1973) and virus DNA and proteins V and VII can be detected within the nucleus between 1 and 2 h (Greber et al., 1997; Matthews & Russell, 1998a). Once inside the nucleus, the genome is targetted to the nuclear matrix (NM), where the TP forms a tight complex with the cellular CAD pyrimidine synthesis enzyme and possibly other NM components (Angeletti & Engler, 1998; Fredman & Engler, 1993). It is interesting that nuclear lamin B, which is a component of the NM, readily binds to p32 (Simos &
Transcription and replication

As noted above, adenovirus transcription can be defined largely as a two-phase event, early and late, respectively occurring before and after virus DNA replication (Fig. 2). Transcription is accompanied by a complex series of splicing events, with four early ‘cassettes’ of gene transcription termed E1, E2, E3 and E4 (Fig. 2).

The E1 gene products can be subdivided further into E1A and E1B. E1A itself has two major components sharing substantial stretches of sequence that are termed 289R (or 13S) and 243R (or 12S), based on the number of amino acid residues. These E1A proteins are primarily concerned with modulating cellular metabolism to make the cell more susceptible to virus replication. At the risk of being oversimplistic, it is convenient to define cellular metabolism as being devoted principally to promoting accurate cell division while retaining specific cellular functions. In so doing, the cell has devised mechanisms to defend this process from external interference and to remove any defective cells. The former operates by invoking the innate and adaptive immune systems (see below), and some of these pathways appear to be regulated via the transcription factor NF-κB, while the latter is mainly carried out by the induction of apoptosis via a number of routes, one being the transcription factor p53 (for a review of p53 pathways see Prives & Hall, 1999).

NF-κB is a nuclear transactivator that is released by proteolysis of an associated inhibitory factor, IκB, in the cytoplasm (Hay et al., 1999b), thus leading to its migration to the nucleus and the activation of NF-κB-responsive genes, among the latter being the E3 gene promoter (Deryckere & Burgert, 1996) (see below). Phosphorylation of IκB by a kinase complex, IKK, appears to be crucial for the proteolysis of IκB.

The protein p53 is a tumour suppressor that regulates the transcription of a variety of genes involved in cell cycle arrest and apoptosis. In normal cells, p53 is present in small amounts, but levels increase in response to genotoxic and other stresses. The regulation of p53 seems to mainly at the protein level, utilizing the cellular protein mdm2, which binds to p53 and acts as a ubiquitin ligase, targetting p53 to the proteosome for degradation. Another cellular protein, p19arf, also contributes to this system by binding to mdm2, blocking its ligase activity and thereby stabilizing p53 (de Stanchina et al., 1998; Honda & Yasuda, 1999; Tao & Levine, 1999; Weber et al., 1999). An additional factor in this regulation has also been uncovered by the finding that p53 can be modified by the small ubiquitin-like modifier (SUMO), leading to activation of p53 (Rodriguez et al., 1999).

E1A proteins interfere with the processes of cell division and with the regulation of NF-κB and p53, and do this by a great variety of strategies involving both direct and indirect interaction with cellular proteins. They can also modulate transcription patterns in favour of virus transcription. A summary of the characteristics of E1A is found in Table 1 and Fig. 3. It should be pointed out that many of the properties ascribed to E1A in Table 1 are based on in vitro studies, whereas the availability of the relevant cellular components in vivo will depend on the nature of the infected cell and its metabolic state. Moreover, other virus gene products can modulate these cellular interactions significantly. For instance,
Table 1. Properties of E1A proteins

Many of the E1A-interacting proteins listed have been mapped to the E1 protein sequences and the locations of those in bold are shown in Fig. 3. It should be noted that no attempt has been made to distinguish between the 243 and 289R formats; they share substantial sequence and differences are more important in the transformation processes. CDK, Cyclin-dependent kinase.

<table>
<thead>
<tr>
<th>Property</th>
<th>Reference(s)</th>
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<tr>
<td>Bind to p21 and related CDK inhibitors, thereby stimulating cell division and growth</td>
<td>Keblusek et al. (1999)</td>
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<td>Bind to cyclins A and E–CDK complexes, which regulate passage to cell DNA synthesis</td>
<td>Faha et al. (1993)</td>
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<td>Bind to the p300/CBP family of transactivators, which play a key role in regulating the transcription of many components of the cell cycle. Cellular supply of CBP appears to be self-limiting and sequestration by E1A probably displaces other factors, thereby modulating normal cellular pathways</td>
<td>Arany et al. (1995), Jones (1995), Dorsman et al. (1997), Lipinski et al. (1999)</td>
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<td>Binding of E1A also modulates the acetyltransferase activities associated with p300/CBP (pCAF), probably by altering nucleosome structure and thereby transcriptional patterns.</td>
<td>Reid et al. (1998), Chakravarti et al. (1999), Hamamori et al. (1999), Perissi et al. (1999)</td>
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<tr>
<td>Binding of E1A to the p300/CBP family inhibits the activity of the transactivator STAT-1, which is required for induction of responses to interferon (see text) and also inhibits the activation of the caspase pathway to apoptosis independent of p53.</td>
<td>Look et al. (1998), McDonald &amp; Reich (1999), Paulson et al. (1999), Putzer et al. (2000)</td>
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<td>Binding of E1A to the RB/p105 family of ‘pocket proteins’, some of which can function as oncoproteins, and in so doing both interfere and promote the activation of a wide range of transcripts.</td>
<td>Zantema &amp; van der Eb (2000)</td>
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<td>One important factor that is released from RB on E1A binding is E2F; this is critical for the synthesis of a range of S-phase components as well as the activation of the adenovirus E2 gene cassette. Binding of E1A may also affect the acetyltransferase activities of RB</td>
<td>Brehm et al. (1998)</td>
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<tr>
<td>E1A also interacts via a PLDLS motif at its C-terminal region with a protein (CBP) which also binds to RB at the same motif and then functions as a transcriptional co-repressor</td>
<td>Meloni et al. (1999)</td>
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<td>E1A activates the promoter of the PCNA gene in HeLa cells; but represses it in rat cells, possibly a factor in the transformation process. This effect appears to be mediated via RB and involves p53</td>
<td>Kannabiran et al. (1999)</td>
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<td>Interact with a multiprotein complex Sur-2, thereby stimulating the transcription of virus genes.</td>
<td>Boyer et al. (1999b)</td>
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<td>Binding to the TATA-box-binding protein (TBP) and TBP-associated protein (TAF) provides a route to transcriptional regulation.</td>
<td>Mazzarelli et al. (1997)</td>
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<tr>
<td>E1A proteins can stimulate the induction of apoptosis by a number of routes. An important one is by promoting the synthesis of p53 and maintaining its integrity. Following binding to pRb, release of the transcription factors E2F and ETS can lead directly to transcription of p53 and to the transcription of the p19ARF gene.</td>
<td>Hale &amp; Braithwaite (1999)</td>
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<td>The product of the latter can interact with mdm2 and thus prevent its interaction with p53 and consequential proteolysis via the ubiquitin/SUMO pathway.</td>
<td>Lin et al. (1994), Honda &amp; Yasuda (1999), Tao &amp; Levine (1999); Weber et al. (1999), Rodriguez et al. (1999)</td>
</tr>
<tr>
<td>In contrast, it has also been shown that transcriptional activation of p53 can be achieved by binding of CBP to a TRAM motif, which, at the same time, prevents the binding of mdm2 and therefore stabilizes p53. The sequestration of CBP by E1A eliminates this interaction. Another route to activation of apoptosis is by sensitization of cells to stimuli such as ionizing radiation, DNA damage, TNF and Fas ligand. This is mediated by inhibiting the IκB kinases, which are critical for the release of NF-κB to the nucleus, and requires the binding of E1A to p300/CBP</td>
<td>Grand et al. (1999 a)</td>
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<td>The stability of p53 can also be maintained by the interaction of E1A with Sug1, a subunit of the proteosome complex that is required for the proteolysis of p53.</td>
<td>Somasundaram &amp; El-Deiry (1997), OConnor et al. (1999)</td>
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<td>E1A can also bind to UBC9, a protein involved in the SUMO enzymatic pathway. Binding to E1A may interfere with the SUMO modification (and stabilization) of cellular proteins such as p53.</td>
<td>Shisler et al. (1996), Shao et al. (1997, 1999)</td>
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the E4 gene products can co-operate with E1A in a variety of ways (Goodrum & Ornelles, 1999; Hall et al., 1998; Yun et al., 1999). The E1B gene product 19K also seems to function co-operatively with E1A and p53 in promoting onco genesis and transformation (Kannabiran et al., 1999), mainly by ensuring that the downstream consequences of cell cycle release do not induce apoptosis.

The E1B 19K gene product is analogous to that from the cellular Bcl-2 gene. This gene product is concerned with prolonging cell survival by interacting and ablating members of the Bax family (whose transcription can be promoted by p53), which induce apoptosis and necrosis (Han et al., 1996) (see below). A number of studies have examined the interaction of the other E1B gene product, 55K, with p53. This interaction
appears to be direct and involves the co-localization of the complex to the cytoplasm (Grand et al., 1999b). Interest in this interaction was stimulated by the development of an E1B-deleted vector that was claimed to act as an oncolytic virus to target tumours with defective p53 genes (Bischoff et al., 1996). Further investigations have shown that, while the interaction reduces the transcription of p53, it also seems to be necessary for virus replication (Ridgway et al., 1997) and for transport of viral RNAs (Horridge & Leppard, 1998). However, the dependence on intact or wild-type p53 is not essential (Harada & Berk, 1999; Vollmer et al., 1999) and, more significantly, the selectivity for replication in defective p53 cells was not apparent when a wide range of cells was tested (Hay et al., 1999a). The involvement of an E4 gene product, E4orf6 (see below), in the interaction of E1B 55k with p53 also influences the stability of p53. In addition, E1B 55k has an effect on late virus mRNA transcription (Harada & Berk, 1999) and functions as an important effector of inflammation in vivo (Ginsberg et al., 1999).

The E2 gene products are subdivided into E2A (DBP) and E2B (pTP and Pol). These provide the machinery for replication of virus DNA (Hay et al., 1995) and the ensuing transcription of late genes, and this is mediated by interaction with a number of cellular factors.

The E3 genes, which are dispensable for the replication of virus in tissue culture, provide a compendium of proteins that subverts the host defence mechanisms (see below) and their properties are summarized in Fig. 4(A). One of these E3 gene products has been termed the adenovirus death protein (ADP), since it facilitates late cytolysis of the infected cell and thereby releases progeny virus more efficiently (Tollefson et al., 1996). The E3 gp19K is localized in the ER membrane and binds the MHC class I heavy chain and prevents transport to the cell surface, where it would be recognized by CTLs. This gene product, in addition, delays the expression of MHC I (Bennett et al., 1999). The E3 proteins RIDαβ and 14.7k inhibit proapoptotic pathways (see below). A recent review on these proteins can be consulted for further information on the molecular mechanisms involved (Wold & Chinnadurai, 2000).

The gene products derived from the E4 cassette (termed orfs 1–6/7) mainly facilitate virus messenger RNA metabolism (sometimes in association with E1B gene products) (Goodrum & Ornelles, 1999; Weigel & Dobbelstein, 2000) and provide functions to promote virus DNA replication and shut-off of host protein synthesis (Halbert et al., 1985) (see Fig. 4B). They are also associated with resistance to lysis by CTLs (Kaplan et al., 1999). Recently, some of the E4 products have been shown to bind to DNA-activated protein kinase, thereby preventing viral DNA concatenation. Since the same kinase is needed for activation of the p53 gene following DNA damage, it has been suggested that such a characteristic could block one of the pathways to apoptosis (Boyer et al., 1999a; Weiden & Ginsberg, 1994). It is significant that many of the characteristics of both the E1B and E4 gene products are related to counteracting the activities of those encoded by E1A, thus E4 orf4 seems to inhibit the E1A activation of the E2F promoter (Mannervik et al., 1999). E4 orf6 has a direct effect on the transactivation of p53 (Boivin et al., 1999) by interacting with E1B 55k when it binds to p53, thereby releasing it to be degraded (Boyer & Ketner, 2000). E4 orf3 also appears to relieve the E1B inactivation of p53 independently (Konig et al., 1999) and to play a role in relocating sites of replication and transcription in the nucleus (pods) (Doucas et al., 1996; Leppard & Everett, 1999). Recent investigations have also shown that p53 can interfere with the binding of the E1A-regulated transcription factor p120E4F, thereby repressing the activities of the E4 promoter (Sandy et al., 2000). A review of the E4 gene products can be consulted for further details (Leppard, 1997).

Adenoviruses also transcribe a set of RNAs (see Fig. 2) that are not translated, termed the VA RNAs, and these play a role in combating cellular defence mechanisms (see below).

Fig. 3. Binding map of proteins to E1A. The locations have been determined on the basis of in vitro studies using deletion and mutational analysis. The abbreviations are explained in Table 1. CR1, CR2 and CR3 are constant regions present in a wide range of adenoviruses. PLDLS and LXEXE etc. are recognition motifs. E2F-1 A.D. homology refers to sequences that can displace E2F-1 from Rb and p300.
Fig. 4. Characteristics of E3 and E4 gene products. Horizontal arrows indicate the relative directions of transcription.

Other characteristics of these early gene products are described below, and a cartoon depicting the effects of some of them on a few cellular pathways is provided in Fig. 5. DNA replication begins from both DNA termini and requires sequences within the ITRs as origins of replication (Hay et al., 1995). Thereafter, late transcription ensues, with five cassettes of transcripts (termed L1 to L5) resulting from a complex series of splicing events. These lead to the production of the virus structural components and the encapsidation and maturation of virus particles in the nucleus. A key player in the control of transcription is the major late promoter (MLP), which is attenuated during transcription of the early genes. However, it should be noted that there is a low basal level of late transcription occurring early in infection, even before the MLP comes into play. After the onset of virus DNA replication, the IVa2 and IX genes are expressed at high levels (see Fig. 2) and transcription via the MLP is fully functional by specific activation. This is accomplished via the IX and IVa2 gene products (Lutz & Kedinger, 1996; Lutz et al., 1997) and is also influenced by effective competition for the limiting transcription factors (Fessler & Young, 1998). The encapsidation process is governed by the presence in the virus DNA of a packaging signal at the conventional left end, which consists of a series of AT-rich sequences (Hearing et al., 1987). These events are accompanied by major changes in the nuclear infrastructure and the permeabilization of the nuclear membrane (Rao et al., 1996; Tollefson et al., 1996). This facilitates the egress of the virus into the cytoplasm and is followed by
the disintegration of the plasma membrane and the release of virus from the cell.

**Defence mechanisms**

Adenoviruses induce only very low levels of morbidity in general and this characteristic initially seemed to make them attractive as gene vectors. However, on closer scrutiny, it becomes evident that a well-orchestrated host defence is the key factor in the suppression of virus spread during infection and, since this could be just as effective against a vector, it becomes important to unravel the complex molecular operations involved in mounting the host defences.

Host cells have a range of strategies to combat any incursion by an intruder; these can be considered as innate and adaptive. With respect to the former, it has recently been established that some epithelial cells release 3–4 kDa antimicrobial peptides termed defensins (Ganz & Lehrer, 1998) and it has been shown that these compounds can provide significant protection from adenovirus infection (Gropp *et al.*, 1999). Indeed, an adenovirus vector expressing a defensin has been utilized to supplement innate defences (Bals *et al.*, 1999). Some tissues, on receiving the appropriate signal (perhaps via the Raf/MAPK pathway; see above), will release multiple chemokines that, in turn, recruit neutrophils and invoke an inflammatory response (Charles *et al.*, 1999; Muruve *et al.*, 1999). Innate defence mechanisms such as recruitment of macrophages, activation of complement and natural killer (NK) cells have been shown to play a significant role in clearing an adenovirus infection *in vivo* (Worgall *et al.*, 1997a, 1999). The transcription factor NF-κB appears to be a key regulator of the innate antiviral response (Ferreira *et al.*, 1999), since it can activate the transcription of cytokines and adhesion molecules,
leading to the production of a range of proinflammatory cytokines and the orchestration of other signalling pathways. It has been claimed that adenovirus infection, especially at high multiplicities, can lead to the activation of NF-κB at early stages of infection (Clesham et al., 1998; Lieber et al., 1998). One mechanism for achieving this could be by the binding of E1A to the p65 subunit of NF-κB, although it is also apparent that this activation can be suppressed by E1B 19K (Pahl et al., 1996; Schmitz et al., 1996). Reference has been made above to the very early induction, possibly by the interaction of the penton base in the virus with cellular integrins, of the Raf/MAPK and other pathways. These may play a role in the activation of NF-κB (Ghoda et al., 1997) as well as in the early release of chemokines (Kuhnel et al., 2000; Muruve et al., 1999) and interferons, which are important components of the innate response to infection. However, the induction of these endogenous genes following infection appears to be quite cell dependent, with human endothelial cells displaying a range of signalling molecules at 24 h, a scenario not seen in human dermal fibroblasts or alveolar macrophages (Ramalingam et al., 1999).

Interferons

The interferons are subdivided into two main classes, type I (containing interferons α and β) and type II (interferon γ). They are cellular proteins ranging in size from 15 to 35 kDa and are released from cells very early after infection by viruses and as a result of other insults to the cell and display a fair degree of cell specificity. In the case of adenoviruses, induction seems to be by interaction with a structural component, since they can be produced by virus particles in the absence of protein synthesis (Reich et al., 1988). The interferons function by binding to cell receptors, thereby activating the cellular Jak/STAT pathways, which lead to STAT complexes being transferred to the nucleus and binding to interferon-response elements (ISREs) on the cellular DNA. The ISREs regulate the transcription of a range of gene products, such as a dsRNA-induced protein kinase (PRK) and a 2′−5′ oligoadenylate synthetase as well as a variety of immunomodulators. These form an impressive array of weapons to combat the intracellular activities of the invading virus; for a recent review see Goodburn et al. (2000). Adenoviruses are generally refractive to interferons, since they have provided themselves with a number of strategies to overcome this assault on their activities. Thus, gene products from E1A downregulate the STAT activators (Look et al., 1998; McDonald & Reich, 1999; Paulson et al., 1999; Leonard & Sen, 1996, 1997).

It has also been claimed (Feigenblum et al., 1998) that adenoviruses induce an interferon-regulatory factor (IRF) at later stages of infection that plays a role in cytopathogenicity. In addition, the VA RNAs (Mathews & Shenk, 1991) bind to and inactivate PRK.

Apoptosis

As another means of combating virus infection, the cell can redirect its metabolism to switch on its apoptosis circuits. Cells have complex mechanisms for ensuring that their integrity is not compromised and they have devised a fall-back strategy to switch on proapoptotic proteins when specific alarm pathways are activated (see Fig. 5). Chief among these is the tumour suppressor p53, which regulates the transcription of genes involved in cell cycle arrest and apoptosis. Among the latter are members of the Bax family (Pearson et al., 2000), which interact with mitochondria and are involved in the induction of caspases, leading to apoptosis. There are other members of this family, such as Bcl-2, which function to inhibit apoptosis and they carry this out by binding to Bfl, an important transcriptional repressor. Bfl promotes cell death (Kasof et al., 1999) by inducing the permeabilization of mitochondrial membranes (Imazu et al., 1999) and releasing cytochrome c, thereby initiating the caspase cascade. Adenoviruses can subvert the operation of this pathway by utilizing virus gene products from its E1 cassette; thus, E1B 19K can inactivate Bax (Han et al., 1996; Ohgi et al., 1999) and has a similar function to Bcl-2 in binding to Bfl; thus counteracting the proapoptotic response of the E1A gene product in activating p53. A parallel mechanism of apoptosis is mediated by TNFα, which is secreted by monocytes and lymphocytes following activation as part of the innate response. This cytokine appears to play a significant part in the elimination of adenovirus vectors (Elkon et al., 1997) and functions by activating cytosolic phospholipase A2 (pL A2), which permeabilizes cell membranes, releasing arachidonic acid (Wolf & Laster, 1999) and initiating the production of prostaglandins and leukotrienes, which also play a role in inflammation (Krajcsi et al., 1996). This pathway is normally modulated by Bcl-2 and its virus analogue, E1B 19K (see above), via downregulation of IkB transcription, thus releasing NF-κB to the nucleus (de Moissac et al., 1999). In this regard, it is intriguing to note that the ability of p53 to induce apoptosis requires the participation of NF-κB (Ryan et al., 2000), implying a degree of co-operative ‘cross-talk’. It is important, however, to note that many of these effects seem to be cell specific: thus, in endothelial cells, Bcl-2 serves to protect the cells from both apoptosis and proinflammatory responses (Badrichani et al., 1999). Another route of TNFα action is by the direct induction of caspases (Kimura & Gelmann, 2000). In contrast, in oligodendrocytes, apoptosis by TNFα appears to be mediated by p53 and involves initiating the JNK signalling pathway (Ladiwala et al., 1999). TNF-induced apoptosis can be ablated by E3 gene products (Lukashok et al., 2000) (see Fig. 5). Other key players in apoptosis are Fas and Fas ligand interactions, and these have been shown to be the major mediators of the elimination of adenovirus vectors from the liver (Chirmule et al., 1999). In this case, the E3 gene products RIDα and RIDβ cause Fas to be removed from the cell surface and degraded (Tollefson et al., 1998). A cellular protein termed
FIP-3 (Li et al., 1999b) has also been implicated in these proapoptotic events. FIP-3 appears to be a scaffolding component of the IKK complex (Ye et al., 2000) and blocks the release of NF-κB by inhibiting the kinase activity of IKK (Fig. 5) (Li et al., 1999b). Adenoviruses modulate these events via the E3 gene product 14.7K, which binds to FIP-3 (Li et al., 1999b) and effectively restores NF-κB transcription and thereby cell survival. Many of these apoptotic mechanisms involve the activation of a range of proteases, such as caspasps, and it has been demonstrated that inhibition of the related ICE-like proteases can boost adenovirus yields (Chiu & White, 1998). A recent investigation has also shown that E1A can induce apoptosis by activation of caspase-8 and is independent of the status of p53 (Putzer et al., 2000). Interferons can also act proapoptotically by inducing caspase-8 (Balachandran et al., 2000), and this can be amplified in infected cells via the dsRNA route (Tanaka et al., 1998). In this case, however, the inhibition of interferon induction by E1A suppresses this apoptotic response. From the above discussion it will be seen that there is a complex interplay of cellular and virus components seeking to control cell survival and promotion of virus replication and spread. Thus, in utilizing adenoviruses as vectors, it is critical to take these factors into account in devising the optimum conditions for delivery and effective expression of the transgene.

**Cellular immune responses**

T cells provide an effective defence via both CD8+ cytotoxic cells (CTLs) and CD4+ helper cells. CTLs function by recognizing a virus antigen in a complex with class I proteins of the MHC on the cell surface. This event releases perforin, resulting in cell lysis, thereby eliminating the infected cells even at an early stage before any virus is assembled and released. The recognition mechanism depends on a virus antigen being available to complex successfully within the ER membrane with an MHC component and then being transported to the plasma membrane. The complex formation is a function of the nature of the cell being infected, as well as the MHC status of the host. It also appears that different virus gene products can provide the target depending on their ability to interact with a particular MHC. It is significant, however, that there appears to be some cross-reactivity of human CTLs in recognizing different adenovirus subgroups (Smith et al., 1998).

Adenoviruses can combat this cellular strategy as described above by utilizing E3 gp19K to retain the MHC antigens in the ER and hence disrupt the recognition process (Kvist et al., 1978). E4 gene products have also been demonstrated to function in the inhibition of T cell cytolysis (Kaplan et al., 1999).

The CD4 helper cells are important in mounting a proliferative response to infection. This is mediated in a similar fashion by recognition of a virus target antigen in association with class II MHC. These helper T cells can thereby stimulate proliferation of B cells to provide immunoglobulins for the humoral response (see below). Very few attempts have been made to examine the adenovirus antigens involved in the initiation of the proliferative response. A study on the lymphoid cells from one individual suggested that either the fibre or IIIa structural polypeptides could be targets (Souberbielle & Russell, 1995). However, a more general investigation noted that proliferative responses to the uncommon Ad35 occurred in individuals without any serological evidence of previous Ad35 infection (Flomberg et al., 1995), implying that CD4+ T cells recognized a conserved antigen. This suggests that this arm of the immune system may play a role in modulating infection with a wide range of serotypes.

**The humoral response**

The humoral response is a major component of the defence strategy of the host and depends on the ability of B cells, elaborating surface immunoglobulins, to recognize a specific epitope on a foreign antigen. This recognition initiates a massive proliferation via T helper cells and thus the release of specific immunoglobulins of various classes into plasma to interact directly with these antigens. Where these are important in the initial interaction with the host cell, virus infection can be neutralized very efficiently. Given the importance of the fibre and penton base in the recognition of the receptors (see above), it is not surprising that adenovirus-neutralizing antibodies are directed against epitopes on these capsid components (Gahery-Segard et al., 1997, 1998; Willcox & Mautner, 1976). However, there are also antigens on the hexon that induce neutralizing antibodies, and these seem to function by aggregating virus particles and thereby inhibiting adsorption. The efficacy of the humoral response in the case of adenovirus gene therapy is very important and depends on the nature of pre-existing immunity as well as the route and target of infection (Harvey et al., 1999).

For humans, there are 51 different adenovirus serotypes, classified on the basis of their specific neutralizing abilities, and protection by humoral antibodies is therefore tightly restricted to a given serotype. Further subdivision into species or subgenera A to F has also been made, using a variety of criteria (Benkô et al., 1999). Type-specific antigens have been described in the fibre that are associated with the trimeric knob and the proximal regions of the stem (Watson et al., 1988; and W. C. Russell, unpublished data). In the case of the hexon, the type-specific epitopes reside, not surprisingly, on the hexon surface, whereas the internal antigens are conserved, being critical in the formation of the capsid structure, and therefore have a very much wider ‘group’ specificity. Group-specific hexon antibodies have been used extensively as general adenovirus diagnostic reagents.

**Adenoviruses as vectors**

Adenoviruses can infect a wide variety of cell types and...
tissues in both dividing and non-dividing cells. This characteristic, together with their relative ease of preparation and purification, has led to their extensive use as gene vectors.

The virus can incorporate only about 2 kb of foreign DNA without significant affects on its stability or its infectivity, and the introduction of longer sequences therefore requires the removal of some or all of the virus genes. There are a range of techniques for constructing recombinant adenoviruses, and these are described in detail elsewhere (Hitt et al., 1997; Tashiro et al., 1999; Zhang, 1999).

Vectors can be utilized for: (i) cancer therapy to deliver genes that will lead to tumour suppression and elimination; (ii) gene therapy, i.e. to deliver genes to tissues to augment defective genes; (iii) supplementary therapy to deliver genes, expression of which will combat disease processes.

First-generation vectors

In the first generation of vectors, the E1 and/or E3 gene cassettes were removed, allowing the introduction of up to 0.5 kb of foreign DNA, often under the control of a heterologous promoter. In the case of the E1 deletions, care was taken to ensure the retention of the ITR and the packaging sequences. Removal of the E1 region had the additional apparent advantage of impairing the transcription of the E2 genes (which are E1 dependent) and consequently the replication of virus DNA and the production of the virus capsid proteins. However, it will be evident from the description of the E1 genes given above that there is also the disadvantage of the cellular environment being much less conducive to vector transcription. The defective E1 viruses could be propagated by infection of 293 cells (Graham et al., 1977), which provide the E1 gene products in trans. Although many of the initial studies in vitro provided much promise, it soon became evident that the expression of the transgene in vivo was only transient and was depressed because of the overwhelming immune response, mounted mainly against the virus capsid antigens as well as the expressed transgene. One of the reasons for this was the observation that many cells harboured E1-like proteins that allowed the E2 genes to function, albeit at reduced levels. In turn, this facilitated virus DNA replication and the synthesis of the late structural antigens and the production of replication-competent adenovirus (RCA). It also became evident that, at higher m.o.i., the E1 dependence of E2 gene transcription could be ablated. Bearing in mind these problems, a number of strategies have been adopted in an attempt to minimize the production of RCA (Hehir et al., 1996; Gao et al., 2000). Furthermore, as described above, removal of the E1B products also effectively disarmed one of the mechanisms for combating proapoptotic defences. In the case of the E3-deleted vectors, there were similar sequelae as a result of the elimination of the E3 gene-mediated defences against host responses (Poller et al., 1996).

Second- and third-generation vectors

The next approach was to construct vectors (using suitable complementing cell lines) with some or all of the E2 genes excised (Lusky et al., 1998; Moorhead et al., 1999) and hence with the capacity to replicate virus DNA and to produce RCAs removed. Generation of RCAs could also be prevented by constructing cell lines that do not contain adenovirus sequences that overlap those in the vector (Fallaux et al., 1998, 1999). Nevertheless, the host immune response was still a major impediment to achieving persistent transgene expression and was particularly evident when repeated infections were attempted. A number of studies confirmed that the infecting recombinant virus itself was sufficient to induce the immune response, perhaps not surprising in view of the early activation of signalling cascades noted above and the potent antigenicity of the capsid components.

Other, rather more sophisticated vectors (third generation) have been constructed by deleting other virus genes (Amalfitano et al., 1998) and the latest of these have all or nearly all of the virus genes removed. These so-called ‘gutless’ vectors (Hardy et al., 1997; Kumar-Singh & Chamberlain, 1996; Lieber et al., 1999; Morsy et al., 1998; Steinwaerder et al., 1999) originally retained only the ITR and packaging sequences and required helper virus and appropriate complementing cells for propagation, followed by careful purification. Nevertheless, there were problems associated with these techniques, mainly due to contaminating helper virus and vector instability. A further development, which prevented the packaging of the helper virus, involved the use of the Cre-lox helper-dependent system (Chen et al., 1996; Hittigan-O’Connor et al., 1999; Ng et al., 1999; Parks et al., 1996; Tashiro et al., 1999).

Other methods to simplify and improve the construction of vectors have been described (He et al., 1998; Mizuguchi & Kay, 1998). A more comprehensive review (Hitt et al., 1997) provides details of most of the different techniques available for construction of vectors. One factor in fabricating these vectors is the need to maintain the vector size for efficient DNA packaging (Parks & Graham, 1997). This has been achieved by using ‘stuffer’ DNA, although the nature of this stuffer segment has been shown to influence transgene expression (Parks et al., 1999b). These latest vectors have increased expression dramatically in vivo (Morral et al., 1999; Morsy et al., 1998; Ji et al., 1999). However, it has become clear that the retention of some of the E4 genes is important in combating the T cell response (Kaplan et al., 1999; Lusky et al., 1999; Yew et al., 1999) and more recent vectors have been modified accordingly (Gorziglia et al., 1999).

An extension of this approach involves the formation of hybrid vectors with adenov-associated virus (AAV) ITRs, which facilitate transgene integration (Lieber et al., 1999; Recchia et al., 1999). A similar strategy has been developed recently by using the long terminal repeats of Maloney leukaemia virus.
(Zheng et al., 2000) and has shown promise both in vitro and in vivo in a model system in facilitating transgene persistence. Hybrids with other viruses such as Epstein–Barr virus and retroviruses have also been developed (Caplen et al., 1999; Tan et al., 1999). Adenoviruses derived from other species (avian, ovine, bovine, canine) have been investigated as vectors for human gene therapy, since they do not normally invoke endemic humoral immunity (Hofmann et al., 1999; Reddy et al., 1999; Zakharchouk et al., 1998). Animal adenovirus vectors have also been used for animal vaccination (Hammond et al., 2000; Rasmussen et al., 1999).

**Strategies for ensuring effective vectors**

The effectiveness of gene therapy is governed in the main by the ability of the vector to be delivered to the relevant tissue and, once there, to express the gene product in appropriate quantities. This, of course, is exactly what the virus has sought to achieve in the course of evolution, and has been demonstrably successful in that adenoviruses are very prevalent without causing excessive morbidity. This seems to have been accomplished by utilizing virus gene products to delay the early innate and immune host defences, thus ensuring that the primary infection produces large amounts of virus. Propagation to other host cells can then be accomplished before the infected host’s full immunological armoury is deployed successfully. Whether adenoviruses are able to adopt other strategies to ensure their continuance, such as persistence or latency in the presence of an immune response, has never been adequately demonstrated, although adenovirus sequences can be detected in a proportion of the lungs from healthy individuals (Eissa et al., 1994; Elliott et al., 1995) as well as patients with pulmonary disease (Keicho et al., 1999) (for reviews see Lukashok et al., 2000; Mahr & Gooding, 1999). However, the facts that adenovirus immunity appears to be long lasting and that antibodies to the common serotypes 2 and 5 can be detected in almost 90% of individuals do suggest that persistence may be a factor in virus survival. This latter characteristic is obviously a desirable feature of an effective vector in some applications, but to achieve such an outcome in vivo characteristic is obviously a desirable feature of an effective vector in some applications, but to achieve such an outcome in vivo would require a much greater understanding of the total spectrum of molecular mechanisms that operate in infection and of the resulting immunity.

**Minimizing immune and apoptotic responses.** In view of the importance of the immune response in relation to transgene persistence, a number of studies have been carried out to unravel the role of the different arms of the immunological repertoire. Most of them have been carried out in model rodent systems, but a few have involved human subjects.

Humoral responses can be mounted, as noted above, with a single immunization (Juillard et al., 1995), but this can be modified to some extent by ensuring the retention of the E3 gene cassette in the vector as well as by treatment of the host with anti-CD4 reagents (Poller et al., 1996). This latter protocol reduced the population of T helper cells, which are needed for the activation of B cells and for the production of neutralizing antibodies. Another study implicated factors other than the capsid antigens in modulating the humoral response. It was shown that deletion of the E4 gene cassette diminished Th2 and B cell activities and it was postulated that an E4 gene product facilitated antigen presentation and the production of IL-6 and IL-8, which are important in B cell maturation (Armentano et al., 1997). Not surprisingly, a number of studies demonstrated that the administration of immunosuppressive agents such as cyclosporin, cyclophosphamide (Smith et al., 1996), FK506 (Ilan et al., 1997), deoxyspergualin (Kaplan & Smith, 1997) and CTLA4 Ig (Jooss et al., 1998b) enhanced the persistence of the transgene product. Induction of tolerance has also been shown to be successful in some cases, leading to significant transgene persistence (Ilan et al., 1996, 1998; Lee et al., 1999b). Another route to immunosuppression was brought into play by the co-administration of an adenovirus vector with another that had a transgene expressing soluble CD8 or CD8 fused to the extracellular regions of a TNFα receptor (Peng et al., 1999). This procedure successfully inhibited the action of TNFα and significantly reduced the humoral antibody responses to both adenovirus and the transgene product. Another, more direct approach to minimizing antibody neutralization was achieved by covalently linking polyethylene glycol (O’Riordan et al., 1999) or a hydrophilic polymer based on N-(2-hydroxypropyl)methacrylamide (HPMA) to the capsid components of the virus (Fisher et al., 2000). This latter procedure also allowed retargeting of the vector. It should be noted, however, that the antibody response to the administration of a virus vector is influenced significantly by the pre-existing antibody status and by the route of administration (Harvey et al., 1999). Some improvement in transgene persistence can be achieved by repeated administration with vectors of different serotypes (Parks et al., 1999a), although this has its limitations in view of the T cell cross-reactivities described above. Nevertheless, in spite of this plethora of techniques available to minimize the humoral antibody response, there is no doubt that the inability to ablate the response effectively remains a major impediment to exploitation of vectors (Benihoud et al., 1999).

In terms of T cells, a number of investigations have indicated that adenovirus gene delivery can elicit a complex panoply of cellular immune responses. CD4+ and CD8+ T cells specific for the transgene product as well as the vector can all be elaborated with variations dependent on the route of administration, the target organ and other factors such as the host genotype (van Ginkel et al., 1997) and development status (Kass-Eisler et al., 1994). In addition, for delivery to the lung, innate immune mechanisms involving the migration of alveolar macrophages seem to be very important (Worgall et al., 1997b).

Apoptosis can also play an important part in minimizing
transgene expression, and this can be combated to a significant extent by using vectors that express Bcl-2 both with and without NF-κB inhibitors (Bilbao et al., 1999a, b; de Moissac et al., 1999; Lieber et al., 1998), the expression of the inhibitors encouraging greater transgene persistence in mouse livers. The role of the E3 14.7K protein in attenuating inflammation was shown neatly by constructing transgenic mice in which this gene was expressed selectively by using a human SP-C promoter. There was a significant difference in lung inflammation and prolonged transgene expression when an E1/E3-deleted vector was administered (Harrod et al., 1998). A systematic investigation of the target proteins for CTLs and their histocompatibility restriction was undertaken in a murine model of liver gene therapy and revealed that the levels of CTL responses to adenovirus antigens and to the transgene product were varied and very dependent on the MHC haplotype of the host. A range of adenovirus antigens were examined in this system (pTP, Pol, DBP, hexon, penton and fibre) and the structural proteins, especially hexon, appeared to be the major targets (looss et al., 1998a). Another survey examined both apoptosis and antibody formation in different strains of mice as a result of infection with an adenovirus vector and also concluded that there were differing responses depending on the mouse strain (Schowalter et al., 1999). An interesting study with nude mice, where the immune system was ablated, also concluded that the persistence of a transgene in mouse lung depended on the nature of the vector backbone and on the host background (Kaplan et al., 1997). These results suggest that the efficacy of therapy with adenovirus vectors will exhibit considerable heterogeneity in human populations.

**Factors that affect delivery of transgenes.** The primary cellular receptors for adenoviruses appear to be distributed so widely in cells that effective and specific delivery to target cells would normally be precluded. On the other hand, a number of tissues and cells express very little, if any, of these receptors (Leon et al., 1998). Thus, the apical surfaces of ciliated airway epithelia, so important in dealing with treatment of cystic fibrosis, do not appear to have CAR available (Walters et al., 1999; Zabner et al., 1997), and the same is true of some primary tumours (Li et al., 1999c; Miller et al., 1998). To permit targeted gene delivery, therefore, novel strategies need to be developed and a number of vectors have been constructed in an attempt to do this. Some of these have bispecific conjugates that can ablate the normal receptor binding and introduce novel tropisms, e.g. by using growth factor receptor (Miller et al., 1998), CD3 (Wickham et al., 1997), fibroblast growth factor (Printz et al., 2000), heparin (Wickham et al., 1996) or gastric releasing peptide (Hong et al., 1999a). In this way, inflammatory vascular endothelial cells exhibiting E selectin can be targeted by complexing an anti-selectin MAb with an anti-FLAG MAb and then attaching this dual antibody to a vector expressing the FLAG epitope (Harari et al., 1999). Another, more direct approach has been to incorporate binding motifs into the C-terminal domain of the fibre protein. This procedure facilitated binding to other cells without altering the endogenous binding, but this technique showed that specific delivery could be obtained in cells where the normal CAR was not expressed (Hidaka et al., 1999). A similar strategy using a variety of ligands proved promising in a model system for mouse gliomas (Staba et al., 2000).

One problem in attempting to produce vectors with novel receptors is the need for simultaneous development of culture cells that would allow good propagation of the vector. A neat approach to dealing with this has been demonstrated by incorporating six histidine residues (a His tag) into the H1 loop of the fibre knob (Krasnykh et al., 1998; Michael et al., 1995) and then using the modified virus to infect human glioma cells successfully (which lack normal receptors), which themselves had been modified to display a single-chain antibody against the His tag (Douglas et al., 1999).

A similar method, using a peptide from influenza virus haemagglutinin inserted into either the fibre or the penton base, was used successfully to infect cells expressing the single-chain antibody ligand (Einfeldt et al., 1999). This approach could, in principle, be developed to construct vectors that have lost their native tropisms through mutation of the receptor-binding site on the fibre (Bewley et al., 1999; Kirby et al., 1999; Santis et al., 1999) and the RGD motif on the penton base (Chiu et al., 1999; Mathias et al., 1998) and therefore have the capacity to infect cells with other specificities. It has also been shown that delivery can be inhibited by protective extracellular matrices (van Deutekom et al., 1999) and that there are also anatomical barriers to overcome (Fechner et al., 1999).

A survey of a range of human adenovirus serotypes has demonstrated that some of them exhibit different and wider host tropisms, indicating that factors other than CAR must also operate. Thus, a chimeric type 2 adenovirus with a type 17 fibre can enhance gene transfer to airway epithelia (Zabner et al., 1999), in contrast with type 2 on its own. Similar use could be made of the properties of adenoviruses of subgroup H; thus, Ad41 binds selectively to differentiated gut enterocytes (Croyle et al., 1998b). Viruses of subgroup D infect primary central nervous system cells more efficiently than do subgroup C (Chillon et al., 1999). One other strategy to ablate the binding properties of the fibre is to use fibreless virus. In this case, infectivity is reduced drastically but entry to cells can still be achieved at low levels via the RGD motif in the penton base (Legrand et al., 1999; Von Seggern et al., 1999). There is additional evidence that virus uptake can be mediated via the penton base alone and that the interaction with integrins can lead to a different route to the nucleus (Hong et al., 1999b).

Other, more non-specific ways of bypassing normal receptor-mediated entry are by transfection with the aid of cationic lipids and polymers and by using calcium phosphate (Alton et al., 1999; Campain et al., 1998; Croyle et al., 1998a; Dodds et al., 1999; Fasbender et al., 1997, 1998; Lee et al.,
1999a; Qiu et al., 1998). Although not strictly a vectorial procedure, the ability of adenovirus to enter cells efficiently has been exploited by condensing a plasmid with polyethyl-

eneimine and then complexing with psoralen-inactivated adeno-

virus (Baker et al., 1997; Bischof et al., 1999; Edgell et al., 1998). A variation, with a simpler technique involving the ability of the virus mu peptide to package and deliver DNA to the nucleus with the aid of liposomes, has also been developed recently (Murray et al., 2000). A peptide derived from adenovirus fibre has recently been shown to target to the nucleolus and may provide a vehicle for gene delivery (Zhang et al., 1999). A combinatorial approach, using adenovirus transduc-

tion and plasmid transfection as well as lipofection, can also lead to enhancement of expression (Dumphy et al., 1999).

Ensuring expression of the transgene. Assuming that there is effective delivery of the transgene to the host cell, the next step in successful expression depends greatly on the efficiency of promoter and enhancer elements. While the backbone E1A promoter may be sufficient in some cases, heterologous promoters have often been inserted to provide better expression. These promoters may be specific to tissues or cells such as prostate (Rodriguez et al., 1997; Yu et al., 1999), muscle (Acasadi et al., 1998; Amalfitano et al., 1999), lung (Harrod et al., 1998), liver (Sandig et al., 1996) or oligodendrocyte (Horwitz et al., 1997). More ubiquitous promoters, such as those derived from the immediate-early human cytomegalovirus (HCMV) promoter, Rous sarcoma virus (RSV) or the phosphoglycerate kinase gene (Millecamps et al., 1999), have also been inserted. The HCMV promoter has been utilized extensively, since it is relatively powerful and requires the insertion of only a small number of bases (380 bp). An interesting comparison (Sallenave et al., 1998) was made of the effectiveness of a series of adenovirus vectors with three different promoters, the adeno-

virus MLP, HCMV and MCMV (mouse cytomegalovirus) promoters. In vitro studies using human and rat alveolar and pulmonary cells showed the MCMV promoter to be very much more effective than MLP, with the HCMV promoter being intermediate. A similar result was obtained in vivo using rat lung. It was also noted that, although the HCMV promoter was much better than the endogenous E1A promoter in inducing persistent expression, this was also influenced by the nature of the vector backbone (Armentano et al., 1997). Careful optimization of the CMV promoter/enhancer sequences can also lead to significant improvements in expression (Massie et al., 1998). The RSV promoter seems to function well in liver (Gorziglia et al., 1999) and has been used in a vector in phase I clinical trials on mesothelioma patients (Sterman et al., 1998) and effectively in ovine vectors (Hofmann et al., 1999). The incorporation of endogenous promoters and enhancers into the vector construct can also improve the transgene expression and persistence dramatically, as demonstrated for apolipo-

protein A (De Geest et al., 2000). A similar result was demonstrated for endogenous liver promoters (Pastore et al., 1999). Another strategy, involving the incorporation into the transgene of tissue ‘silencer’ elements in conjunction with ubiquitous promoters to drive tissue-specific expression in neuronal cells, has been implemented successfully (Millecamps et al., 1999).

From the above discussion, it will be evident that, by modification of receptor-binding characteristics coupled with a judicious use of promoters/enhancers, much more effective delivery and expression of a transgene can be achieved.

Application of adenovirus vectors

The ability of adenovirus vectors to deliver and express genes at high yields, especially in vitro, has been amply demonstrated over the last 15 years and has been well documented. However, the supremacy of the immune response in vivo has been a limiting factor in the practical development of vectors. Thus, it is critical to control or to suppress the immune response to the vector and the transgene where persistent expression is needed in the case of supplementing the activity of a faulty gene. In contrast, cancer therapy may benefit from the induction of a vigorous immune response. With a better understanding of the molecular and immuno-

logical factors that operate in vivo, great strides have been made over the last few years in the construction of more effective vectors, and some of these will be described here.

Gene therapy in the treatment of cancer

A variety of techniques have been adopted to suppress or eliminate tumour cells, the approach in each case depending largely on the type and location of the tumour. Most of the vectors have been developed by using in vitro models of the tumours and then by testing in the appropriate animal, sometimes using tumour-transplantation methodologies. A number of these approaches have now moved on to clinical trials. The therapies in use can be divided into three groups: (i) tumour suppressors, (ii) oncolytic and sensitizing drug therapy and (iii) vaccines.

(i) Tumour suppressors. Mutations in the p53 gene that lead to loss of function have been implicated in the development of a wide variety of human tumours (Wills et al., 1994). To remedy this defect and to induce apoptosis in the tumour cells, a number of vectors incorporating wild-type p53 have been constructed. Initial studies, using appropriate tumour cell lines and then animal model systems, demonstrated the efficacy of these approaches with anaplastic thyroid cancer (Blagosklonny et al., 1998), human malignant gliomas (Cirielli et al., 1999; Li et al., 1999a) and breast cancer (Putzer et al., 1998). In some cases, combination with an immunomodulatory gene such as IL-2 (Putzer et al., 1998) or with a cytotoxic drug such as adriamycin (Blagosklonny et al., 1998) proved more effective. Clinical trials testing the efficacies of these vectors in the treatment of lung, head, neck and liver cancers are under way. However, one of the problems inherent in this approach is the
desirability of efficient targetting of the vector, and direct inoculation via the hepatic artery for treatment of liver cancer has been explored in a mouse model (Anderson et al., 1998). More recent investigations have illustrated dramatically the importance of the ARF–mdm2–p53 interactions in regulating p53 expression, and the discovery of a range of mutations in ARF and in related transcription factors such as Taaïst (Maestro et al., 1999) in a variety of tumours (Eischen et al., 1999; Sanchez-Cespedes et al., 1999; Taniguchi et al., 1999) have suggested that vectors expressing other components of the p53 pathway might be equally productive. Other approaches to induce apoptosis have been explored, and these have involved inhibiting cyclin-dependent kinases, which are critical to the cell cycle. Indeed, one of these, p16, has been shown to be defective in many human tumour cell lines, and vectors expressing p21, p15 and p16 have shown promise in model tumour systems (Tsao et al., 1999). The use of vectors expressing proapoptotic proteins such as Fas ligand and caspase-8 has been limited by the difficulty of production of the vectors. However, the construction of complementing cell lines expressing adenovirus E3 14.7k or the poxvirus serpin gene CrmA has recently provided a route for good production and exploration of their properties (Bruder et al., 2000). Another intriguing strategy to encourage apoptosis has been the use of ribozymes such as anti-H-ras for bladder cancer (Irie et al., 1999), anti-Bcl-2 for prostate cancer (Dorai et al., 1999) and anti-HER2 for breast cancer (Suzuki et al., 2000).

(ii) Oncolytic and sensitizing drug therapy. Direct application of wild-type adenovirus to tumours was attempted soon after their discovery in the 1950s, but only local effects were demonstrated, and it was not until 1996 that it was claimed that an adenovirus that had a mutation in E1B 55k would replicate selectively in p53-defective tumour cells (Bischoff et al., 1996) and therefore could function as an oncolytic virus. This led to the commercial development of the mutant (Onyx 015) and, although a number of publications have shown convincingly that the original premises did not hold up (Hay et al., 1999a; Ridgway et al., 1997; Vollmer et al., 1999), it is still claimed that the virus is effective by intravenous administration in treatment of some tumours (Heise et al., 1999). Combination with standard chemotherapy also looks promising (Heise et al., 1997), and phase 3 clinical trials for head and neck tumours using Onyx are under way. Moreover, a recent study using the E1B-deleted virus in parallel with an adenovirus vector expressing IL-2 has shown complete regression of a p53-deficient pancreatic tumour in a mouse model (Motoi et al., 2000).

Another strategy that has been developed for the selective elimination of tumour cells is to deliver a prodrug enzyme via a vector into target cells and then to administer a non-toxic drug that can be converted into a cytotoxic agent in situ (Crystal, 1999). Herpes simplex virus thymidine kinase (HSV-tk) has been used extensively as a so-called suicide gene, since administration of ganciclovir will be followed by its phosphorylation by HSV-tk to yield a chain terminator for DNA synthesis. This technique has been used in situ for head and neck tumours (Goebel et al., 1998) and phase 1 trials have been completed for malignant mesothelioma (Sterman et al., 1998) and for prostate carcinoma (Herman et al., 1999). A vector with both E1 and E4 deleted has been constructed recently for delivery of the HSV-tk gene (Lanuti et al., 1999), apparently with better results. Cytosine deaminase (CD) has also been used in colon carcinoma as a suicide gene in an adenovirus vector, with co-administration of 5-fluorocytosine (Hirschowitz et al., 1995). A fusion gene of HSV-tk and CD has also been inserted into vectors to treat prostate carcinoma (Blackburn et al., 1999). A trimodal therapy involving a double suicide vector of HSV-tk and CD combined with radiotherapy has provided very effective tumour reduction in a cervical carcinoma xenograft model (Rogulski et al., 2000). Another interesting approach has been to co-administer with the CD vector another containing the gene for uracil phosphoribosyltransferase. In a rat tumour model, this increased the sensitivity of the system very significantly (Adachi et al., 2000). A so-called ‘bystander effect’ appears to amplify the cytotoxicity with these therapies (Zhang & DeGroot, 2000).

Vectors containing suicide genes have been complemented with vectors delivering a variety of cytokines in an attempt to boost the in situ cytotoxicity (Cao et al., 1998).

In all these systems, it was evident that the efficacy of treatment could be enhanced considerably if targetting could be made more specific, and tissue-specific promoters have been incorporated into vectors to facilitate this (Hart, 1996). Applications to breast cancer (Manome et al., 1994), liver cancer (Kaneko et al., 1995) and melanoma (Siders et al., 1998a) have been described. However, although specificity was obtained in vivo, results were quite often disappointing because of relatively low promoter activity. In an attempt to improve this, an ingenious approach has been developed using the Cre-lox system and a tumour-specific antigen in a model system, with promising results (Kijima et al., 1999).

Reference should also be made to the importance of retaining the E1A genes in vectors in these cases because of their ability to promote p53 transcription and to enhance the sensitivity of tumour cells to cytotoxic agents (Brader et al., 1997; Cook et al., 1999; Wildner et al., 1999) and to radiation therapies (Martin-Duque et al., 1999).

(iii) Vaccines. Strategies to invoke anti-tumour cell immunity have been explored using vectors by introducing a variety of immunomodulatory genes and/or tumour-specific antigens. Many cytokines can be effective in this way: thus, IL-2 can induce CTLs, enhance NK cell activity and promote tumour-infiltrating lymphocytes, and high doses of recombinant IL-2 and IL-2-expressing vector have been successful in reducing tumour load in animal models. Nevertheless, problems of toxicity have become apparent (Toloza et al., 1996) and later
developments concentrated on the more-directed delivery of other cytokines such as IL-12 (Bramson et al., 1996; Gambotto et al., 1999; Mazzolini et al., 2000; Siders et al., 1998b), sometimes in combination with IL-2 (Addison et al., 1998) and tumour antigen (Hirschowitz & Crystal, 1999). Intratumoural injection of vectors expressing IL-2 or IL-12 in combination with a vector expressing lymphoactin have been successful in a murine breast cancer model system (Entage et al., 1999). The recognition that many tumours exhibit tumour-specific antigens encouraged the use of vectors expressing these antigens as a means of boosting anti-tumour immunity and led to a trial on metastatic melanomas using adenovirus vectors expressing either MART 1 or gp100 melanoma antigens (Rosenberg et al., 1998). The results demonstrated that high doses of the vector could be administered safely, but that the immune response to the vector ablated any longer-term anti-tumour response. In the case of a model system of colorectal cancer (Li et al., 1997), expression of a tumour antigen via an adenovirus vector resulted in significant tumour regression and induction of immunity to further tumour challenge. Another promising approach to boosting anti-tumour immunity was modelled on the ability of dendritic cells to present antigens effectively in vivo. It has been proposed that one useful strategy would be to isolate dendritic cells from a patient and then to modify them by infection with an adenovirus vector expressing the appropriate tumour antigen, followed by readministration to the patient in combination with standard therapies (Crystal, 1999). Studies with human dendritic cells have shown that dendritic cells modified with adenovirus vectors are not perturbed in terms of their maturation and function (Rea et al., 1999; Zhong et al., 1999) and significant reduction of lung metastases was achieved with murine dendritic cells (Wan et al., 1999). A murine dendritic cell model of melanoma also showed a significant boost to anti-melanoma immunity using an adenovirus vector (Tuting et al., 1999). Considerable improvement in the efficiency of targetting of the vector to dendritic cells can be obtained by using a bispecific antibody to redirect the virus to CD40 receptors on the cells (Tillman et al., 1999). This technique also facilitated the maturation of the cells and thereby enhanced their immuno-stimulatory characteristics. Vectors expressing CD40 ligand have also been introduced directly into tumour cells and facilitate antigen presentation (Crystal, 1999). Other strategies to do this involved the vector expression of granulocyte–macrophage colony-stimulating factors (Ozawa et al., 1999) and increasing the tolerogenicity of the dendritic cells with a vector encoding TGF β1 (Lee et al., 1999b). A recent study has shown that dendritic cells can be infected with a vector independent of their CAR status and that the cells can elicit the appropriate CTL responses (Linette et al., 2000).

**Gene therapy for genetic diseases**

Cystic fibrosis is a relatively common, inherited, recessive disease caused by mutations in the CFTR gene that result in poor chloride ion conductance and increased sodium ion uptake. Since the defect is manifested primarily in the lung, adenoviruses, with their apparent propensity to infect this organ, would appear to be the vector of choice to deliver the therapeutic gene. However, a multiplicity of investigations have demonstrated that there are many barriers to successful transgene expression. Among these is the inability of the standard Ad2/5 vectors to infect the well-differentiated airway epithelial cells and alveolar macrophages, mainly due to the lack of CARs (Kaner et al., 1999; Pickles et al., 1998; Walters et al., 1999). Attempts have been made to improve uptake by using cationic lipids and calcium phosphate co-precipitates (Alton et al., 1999; Fasbender et al., 1997, 1998; Lee et al., 1999a). A more promising approach has been the construction of a chimeric adenovirus vector with serotype 17 fibre, which displays an increased binding to airway epithelial cells (Zabner et al., 1999). Another barrier to successful transgene expression was the specific pulmonary-associated T helper cell response that was the specific pulmonary-associated T helper cell response (van Ginkel et al., 1997), and later studies have shown the importance of retaining E4 genes in the vector to counteract this assault (Armentano et al., 1997; Chirmule et al., 1998; Lusky et al., 1999; Yew et al., 1999). A further, natural inhibitor is derived from the ability of airway epithelia to release antimicrobial peptides with anti-adenovirus properties (Gropp et al., 1999), and a study of bronchoalveolar lavage fluid has indicated the presence of adenovirus inhibitors in addition to neutralizing antibodies (Bastian & Bewig, 1999). Many of these factors probably account for the results from human trials with earlier vectors in which, although efficacy of transfer was demonstrated, the overall efficiency was low and expression of the transgene was of relatively short duration (Zuckerman et al., 1999). It remains to be seen whether the later vectors can improve the efficiency and persistence of the transgene.

Considerable effort using adenovirus vectors has been devoted to trying to repair the gene defects responsible for muscular dystrophy. In a mouse model system, vectors encoding dystrophin can be delivered to muscle fibres with some improvement of function, but this is only transient, because of a potent immune response against the vector and transgene (Yang et al., 1998; Yuasa et al., 1998). Attempts to circumvent this by using improved vectors (Kumar-Singh & Chamberlain, 1996) and utrophin (a homologue of dystrophin) (Gilbert et al., 1999) showed better transgene persistence. One of the barriers to effective gene transfer relates to the lack of receptors for the vectors being used, and a vector with a polylsine-modified fibre improved uptake into muscle cells significantly (Bouri et al., 1999). It has been shown that the immune response in the muscle fibre cells appears to be mediated via dendritic cells, and it has been suggested that AAV vectors may be better vehicles for delivery of the transgene (Looss et al., 1998c). A canine model system has also demonstrated the significance of the immune response, since treatment with cyclosporin after adenovirus delivery of
dystrophin gives a more sustained improvement of muscle function (Howell et al., 1998).

A number of vectors have been constructed to alleviate gene defects in other tissues, and similar problems in terms of delivery have been encountered.

**Supplementary therapy**

With an increasingly ageing population, therapy for human neurodegenerative diseases such as Parkinson’s offers a major challenge. Adenoviruses, with their ability to infect postmitotic cells coupled with a potentially high transduction efficiency and low pathogenicity in the immunologically privileged site of the central nervous system, should provide effective vectors for neuronal gene therapy. Two major strategies have been examined for delivering therapeutic genes. One involves direct intracerebral injection of the vector and the other uses *ex vivo* gene therapy, where cells can be modified *in vitro* by vector infection and then transplanted into the relevant areas of the brain (Barkats et al., 1998). Neuroprogenitor cells (Fisher, 1997) and human astrocytes (Ridet et al., 1999) can apparently provide autologous cellular vehicles for *ex vivo* modification and expansion. A tetracycline-regulated adenovirus vector expressing tyrosine hydroxylase, a rate-limiting enzyme in the synthesis of dopamine, has shown considerable promise in model systems using the *ex vivo* technique (Corti et al., 1999a, b). In an attempt to utilize adenovirus vectors to alleviate Huntington’s disease, a construct expressing brain-derived neurotrophic factor was found to give promising results in a rat model system (Bemelmans et al., 1999). Reference has also been made above to the use of neuronal silencer elements in adenovirus constructs in restricting expression to neuronal cells, with the expectation that such a strategy would avoid the side-effects due to ectopic expression of transgenes (Millecamps et al., 1999).

Over the last few years, there has been considerable progress in our understanding of arthritic diseases and the role that cytokines play in invoking the inflammatory processes that occur in joints and synovial fluids. A major discovery has been the role that TNFα plays in the induction of rheumatoid arthritis, amply demonstrated by the success of clinical trials with TNF antibody (Maini et al., 1999) and TNF receptor (Franklin, 1999). Adenovirus vectors have been extremely useful in unravelling the significance of some of the cytokines and their pathways in this disease process. Thus, direct delivery of TNFα receptor and cytokine IL-1 using adenovirus vectors has shown synergistic benefits, both direct and distal, in a rat model system (Ghivizzani et al., 1998). Similarly, TNFα receptor showed significant synergy with an Epstein–Barr virus homologue of IL-10 in an animal model (Kim et al., 2000; Lechman et al., 1999). Use of a vector expressing IL-4 has demonstrated that this cytokine can provide considerable protection for cartilage from inflammatory processes (Lubberts et al., 1999). In contrast, vector delivery of IL-12 accelerated the disease process (Parks et al., 1998). A significant advance was made by the observation that vector expression of an inhibitor of NF-κB inhibited the production of TNFα in macrophages (Foxwell et al., 1998), and later investigations showed that inhibition of NF-κB was accompanied by the inhibition of proinflammatory cytokines, but not the main inflammatory mediators like IL-10 (Bondeson et al., 1999a, b). These results therefore pinpointed the important role of NF-κB in inflammation and as a therapeutic target. Nevertheless, it should be pointed out that TNFα production did not appear to be NF-κB-dependent in some other cells, such as monocytes (Hayes et al., 1999).

**Other applications**

Adenoviruses have been useful vectors for the production of a number of proteins for more-detailed molecular analysis, and the reader is referred to the very comprehensive review by Hitt et al. (1997). Adenovirus vaccines have been tested thoroughly for safety, as a result of problems with adenovirus respiratory disease in the American military (Chanock et al., 1966), and this has facilitated the development of recombinant adenovirus vaccines for human immunodeficiency virus (Bruce et al., 1999) and rabies virus (Matthews et al., 1999; Yarosh et al., 1996). A comprehensive review of earlier vaccine developments is available (Graham & Prevec, 1992).

This review has only touched on a relatively small number of the applications of vector technology and is certainly not comprehensive, but is illustrative of the considerable progress that has been made; Table 2 provides further examples of applications.

**Safety considerations**

The protocols for developing a virus vector for clinical use invariably employ a series of steps that begin with a study of infection of appropriate cells *in vitro*, followed by application to an animal model system of some kind. This latter step can bring to light some of the problems inherent in delivery to the site of interest and in the expression and persistence (where that is required) of the transgene. Very often, the efficiency of delivery has been found to be poor and the expression of the transgene to be only transient, although some of these effects may be a function of a different distribution of receptors in the model systems. With a greater understanding of the nature of the responses *in vivo*, many of these problems can be alleviated to some extent, as described above, by constructing vectors that improve targeting and minimize immune responses to both the vector and the transgene (Miller & Whelan, 1997). Nevertheless, in the course of *in vivo* studies, it has become apparent that there can be acute injury and inflammation of infected tissues quite independent of the possibility of helper virus replication. This was particularly apparent in the liver (Lieber et al., 1997), where it was shown that a vector can induce production of chemokines within 1 h, even with psoralen-inactivated virus (Muruve et al., 1999). This was followed by an influx of neutrophils into mouse liver and, with
Table 2. Some recent vector applications

<table>
<thead>
<tr>
<th>Site/disease</th>
<th>Transgene(s)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Tumours</td>
<td></td>
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<tr>
<td>Prostate</td>
<td>p16</td>
<td>Steiner et al. (2000)</td>
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<tr>
<td></td>
<td>PKC</td>
<td>Fujii et al. (2000)</td>
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<td></td>
<td>Cytolytic virus</td>
<td>Rodriguez et al. (1997)</td>
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<td></td>
<td>Fas ligand</td>
<td>Hedlund et al. (1999)</td>
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<tr>
<td>Colon</td>
<td>GM–CSF/IL-2</td>
<td>Diaz et al. (1998)</td>
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<tr>
<td></td>
<td>CD suicide gene</td>
<td>Topf et al. (1998)</td>
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<tr>
<td></td>
<td>APC</td>
<td>Shih et al. (2000)</td>
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<tr>
<td></td>
<td>Lymphotactin + suicide</td>
<td>Ju et al. (2000)</td>
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<tr>
<td>Cervix</td>
<td>p21</td>
<td>Tsao et al. (1999)</td>
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<tr>
<td></td>
<td>Papillomavirus p21</td>
<td>He et al. (2000)</td>
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<tr>
<td>Ovary</td>
<td>Stomatostatin receptor</td>
<td>Rogers et al. (1999)</td>
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<tr>
<td>Endometrium</td>
<td>Cytolytic virus/IL-6</td>
<td>Rancourt et al. (1999)</td>
</tr>
<tr>
<td>Gliomas</td>
<td>p53/p21</td>
<td>Ramondetta et al. (2000)</td>
</tr>
<tr>
<td>Carcinomaembryonic antigen-producing cancer</td>
<td>Caspase-3/Fas ligand</td>
<td>Shinoura et al. (2000)</td>
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<tr>
<td>Other conditions</td>
<td></td>
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</tr>
<tr>
<td>Autoimmune diabetes</td>
<td>Adenovirus E3</td>
<td>von Herrath et al. (1997)</td>
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<tr>
<td>Glycogen storage disease II</td>
<td>Glucosidase</td>
<td>Amalfitano et al. (1999)</td>
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<tr>
<td>CNS conditions</td>
<td>(via astrocytes)</td>
<td>Ridet et al. (1999)</td>
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<tr>
<td>Factor VIII deficiency</td>
<td>Factor VIII</td>
<td>Balagu et al. (2000)</td>
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<tr>
<td>OTC deficiency</td>
<td>OTC</td>
<td>Batshaw et al. (1999)</td>
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<tr>
<td>Liver graft</td>
<td>Bcl-2</td>
<td>Bilbao et al. (1999a)</td>
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<tr>
<td>Leishmania infection</td>
<td>IL-12</td>
<td>Gabaglia et al. (1999)</td>
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<tr>
<td>Tay–Sachs disease</td>
<td>Hexasaminidases</td>
<td>Guidotti et al. (1999)</td>
</tr>
<tr>
<td>Motor neurone disease</td>
<td>Neurotrophic factor</td>
<td>Haase et al. (1999)</td>
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</table>

high virus inputs, to hepatic necrosis and apoptosis. It seems evident that the innate system, possibly via NF-κB mediation, is a major player that must be taken into account even with so-called gutless vectors, and consideration could therefore be given, in appropriate cases, to concomitant introduction of a vector that expresses inhibitors of this pathway. This problem has been highlighted by the recent tragic death of a patient undergoing adenovirus vector therapy for a defect in the ornithine transcarbamylase (OTC) pathway. The preliminary enquiries appear to point to a massive activation of innate immunity followed by systemic inflammation (Marshall, 1999). It may be significant that a large dose of virus was given in an effort to get enough functioning OTC genes into the liver. Apparently, this dramatic reaction had not been noted before, and it was suggested that this response was an unusual one related to this particular individual’s genetic repertoire. However, it has now also come to light that a substantial number of adverse effects had not been reported adequately in some clinical trials. These events highlight the necessity to carry out trials using monitoring procedures that have the confidence of the public as well as all of the parties that are directly involved. As indicated above, use of animal model systems had indicated previously that host responses to vector were very dependent on host genotype, and this unfortunate incident emphasizes the need to understand the basic factors that control host–vector responses.

It should be noted that there have been a number of reports of inflammatory responses in animals, not only following vector administration to the liver. Thus, airway neurogenic inflammation was apparent in a rat cystic fibrosis model system, but this could be alleviated by pharmacological methods (Piedimonte et al., 1997).

A significant improvement was seen by utilizing gutless vectors in a rat model system for leptin delivery, where liver toxicity, inflammation and cellular infiltration were reduced significantly compared with an E1-deleted vector (Morsy et al., 1998). However, the problems and considerable costs of scaling up, purification and production of these vectors are not insignificant. The need for careful evaluation of long-term inflammatory responses in human therapy has been underlined further by the report of chronic brain inflammation following the successful inhibition of a glioma using cytotoxic gene therapy (Dewey et al., 1999). In this report, persistence and expression of the HSV-tk gene could be found in the brain...
some 3 months later and co-incident with the presence of a variety of inflammatory markers. In contrast, a study examining the toxicity associated with suicide gene therapy in prostate cancer found that toxicity was not a major factor, even with multiple applications (Shalev et al., 2000). The importance of the route of vector delivery has been demonstrated by the finding that vascular inflammation occurred in a rabbit vascular model when delivery was by the intraluminal route, but was greatly reduced when the adventitial route was used (Schneider et al., 1999). Another, perhaps surprising finding in a porcine model was that direct myocardial administration of a vector proved successful without any apparent inflammatory responses, although the experiment was continued for only 28 days and therefore longer-term chronic toxicity was not assessed (Patel et al., 1999). A study in sheep, in contrast, led to the conclusion that administration of adenovirus vectors in utero was accompanied by inflammatory and fibrotic responses (Iwamoto et al., 1999).

Future developments

Adenovirus vectors have been in vogue for over 15 years and the early enthusiasm in their therapeutic application has now been tempered by the realization that there is, as yet, no evidence of significant clinical success. As noted above, the protective responses of the host had been underestimated and it is only in the last few years has there been some understanding of the complexities of virus expression and the host responses. In this connection, it should be noted that most of the clinical trials presently under way have utilized the earlier first- and second-generation vectors. This rather negative scenario has been compounded by the news of the death earlier first- and second-generation vectors. This rather negative scenario has been compounded by the news of the death of the patient undergoing vector therapy. Nevertheless, great progress has been made and lessons should have been learned. Principal among these is the necessity to explore the many factors involved in vector administration in depth before venturing into clinical application. Thus, as well as trying to understand the molecular nature of both the innate and active immune responses in the context of genetic background, the target organ, the route of administration etc., the possibility of pharmacological and vectorial supplements will need to be examined. This, of course, implies that much longer development protocols will be required and much greater patience will need to be exercised by the institutions and companies that invest in this technology. Nevertheless, adenovirus vectors offer great promise and should not be abandoned in the light of these early mishaps in clinical trials.

The author is indebted to his colleagues, who supplied advice on both the text and the compilation of the figures: viz. Rick Randall, Ron Hay, Martin Ryan, Bernie Precious and Alex Houston. Thanks also to David Matthews, who supplied the basis of Fig. 1, and Phil Gallimore and Andy Turnell for Fig. 3.

References


production of adenovirus vectors expressing apoptotic transgenes. Human Gene Therapy 11, 139–149.


Antimicrobial peptides of vertebrates. Current Opinions in Immunology 10, 41–44.


Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knee with experimental arthritis has local and distal anti-arthritic effects. Proceedings of the National Academy of Sciences, USA 95, 4013–4018.

The 90-kDa ribosomal S6 kinase (p90rsk) phosphorylates the N-terminal regulatory domain of IxBα and stimulates its degradation in vitro. Journal of Biological Chemistry 272, 21281–21288.

Tumor reduction in vivo after adenoviral mediated gene transfer of the phenotype of mdx mouse muscles. Human Gene Therapy 10, 1299–1310.

Role of the type 5 adenovirus gene encoding the early region 1B 55-kDa protein in pulmonary pathogenesis. Proceedings of the National Academy of Sciences, USA 96, 10409–10411.


Adenovirus early region 1A protein binds to mammalian SUG1 – a regulatory component of the proteasome. Oncogene 18, 449–458.

Definition of a major p53 binding site on Ad2E1B54K protein and a possible nuclear localization signal on the Ad12E1B54K protein. Oncogene 18, 955–965.

The role of the adenovirus protease on virus entry into cells. EMBO Journal 15, 1766–1777.

The role of the nuclear pore complex in adenovirus DNA entry. EMBO Journal 16, 5998–6007.


Therapeutic benefit of ciliary neurotrophic factor to cytokine-activated vascular endothelium via E-gp55 (E2) gene protects pigs against classical swine fever. Human Gene Therapy 6, 5333–5344.


Transcriptionally targeted gene therapy. Current Topics in Microbiology and Immunology 213, 19–25.


Adenovirus E4 open reading frame 4-induced dephosphorylation inhibits complexes with dynein and a small GTPase.

Adenovirus inhibitor of tumor necrosis factor alpha-induced apoptosis

In vivo radiosensitizing effect of the adenovirus E1A gene in murine breast cancer cells to ganciclovir.

Simplex virus thymidine kinase gene and confer sensitivity of human adenoviruses.

In vitro and in vivo biology of recombinant adeno viruses vectors with E1, E1/E2A, or E1/E4 deleted.

Regulation of adenovirus-mediated transgene expression by the viral E4 gene products: requirement for E4 ORF3.

Properties of the adenovirus IVa2 gene product, an effecter of late-phase-dependent activation of the major late promoter.

The product of the adenovirus intermediate gene IX is a transcriptional activator.

Cooperation of the transcriptional coactivators CBP and p300 with Stat6.

Twist is a potential oncogene that inhibits apoptosis.

Immune evasion by adenoviruses.

Infliximab (chimeric anti-tumour necrosis factor alpha monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial.

Adenovirus E4 open reading frame 4-induced dephosphorylation inhibits E1A activation of the E2 promoter and E2F-1-mediated transactivation independently of the retinoblastoma tumor suppressor protein.

Enhancer sequences of the DF3 gene regulate expression of the herpes simplex virus thymidine kinase gene and confer sensitivity of human breast cancer cells to ganciclovir.

Gene therapy death prompts review of adenovirus vector.

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Gene therapy death prompts review of adenovirus vector.


Segerman, A., Mei, Y. F. & Wadell, G. (2000). Adenovirus types 11p and 35p show high binding efficiencies for committed hematopoietic cell lines and are infective to these cell lines. Journal of Virology 74, 1457–1467.


Published ahead of print (11 August 2000) in JGV Direct as DOI 10.1099/vir.0.17217-0