Eur Respir J 1998; 11: 492–497
DOI: 10.1183/09031936.98.11020492

European Respiratory Journal
ISSN 0903 - 1936

SERIES 'CELL BIOLOGY OF RESPIRATORY MUSCLES'
Edited by M. Decramer and M. Aubier
Number 3 in this Series

Respiratory muscles as a target for adenovirus-mediated gene therapy

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ABSTRACT: The protein dystrophin is absent in the muscles of patients with Duchenne muscular dystrophy (DMD) as well as dystrophin-deficient mice with muscular dystrophy (mdx mice). The mdx mouse diaphragm closely resembles the human DMD phenotype and thus provides a useful model for studies of dystrophin gene replacement.

Recombinant adenovirus vectors (AdVs) hold promise as a means for delivering a functional dystrophin gene to muscle. As an initial step toward this goal, we have determined the efficiency and functional consequences of AdV-mediated reporter gene transfer to the diaphragm in both normal and mdx adult mice.

At 1 week after AdV administration, there was a high level of transgene expression in the diaphragm. One month later, however, elimination of transgene expression was observed along with a significant decrease in force production by both normal and mdx diaphragms. Immunosuppression with cyclosporine did not augment the level of transgene expression, but a beneficial effect on diaphragm force-generating capacity was observed in both groups of animals. In order to further elucidate the cellular mechanisms underlying these findings, the effects of AdV gene inactivation (by ultraviolet (UV) irradiation) and interference with host T-lymphocyte subsets were examined. Both UV-inactivation of AdV and CD8+ T-cell deficiency were found to significantly alleviate AdV- induced reductions in diaphragm force-generating capacity. Brief (2 day) administration of a neutralizing antibody against host CD4+ T-cells also produced a trend towards mitigation of AdV-induced contractile dysfunction. In addition, transgene expression one month after AdV delivery was significantly enhanced with inhibition of either CD4+ or CD8+ T-cell function.

The data suggest two major sources of reduced force generation after recombinant adenovirus vector-mediated gene transfer to muscle: 1) a cytotoxic component associated with recombinant adenovirus vector transcriptional activity; and 2) an immune-based component of more delayed onset that is primarily dependent upon CD8+ T-cell activity. These results have important implications for the design of future generation vectors and the potential need for immunosuppressive therapy after recombinant adenovirus vector mediated dystrophin gene transfer to Duchenne muscular dystrophy patients.

Eur Respir J 1998; 11: 000–000.

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Keywords: Adenovirus
diaphragm function
Duchenne muscular dystrophy
dystrophin
gene transfer
immunosuppression

Received: September 29 1997
Accepted after revision October 12 1997

This work was supported by grants from the Medical Research Council of Canada, the Muscular Dystrophy Association of Canada, and the Association Pulmonaire du Quebec.

Patients with Duchenne muscular dystrophy (DMD) generally succumb to respiratory failure by the third decade of life due to disease involvement of the diaphragm and other respiratory muscles [1]. Although pharmacological treatment with corticosteroids may briefly delay disease progression [2], definitive therapy would require replacing the missing gene product, dystrophin [3]. Dystrophin is a large cytoskeletal protein that is normally present on the cytoplasmic aspect of the myofibre cell surface membrane [3]. Current evidence suggests that myofibres lacking dystrophin are abnormally susceptible to contraction-induced damage of the sarcolemma [4, 5], which secondarily leads to muscle fibre dysfunction [6, 7], necrosis and eventual replacement of the lost fibres by adipose and connective tissue. Therefore, the muscle weakness found in DMD patients is due to both myofibre loss and impaired contractile function in the surviving myofibre population [6]. Gene therapy approaches in DMD should ideally be capable of reversing and/or preventing these changes without entailing significant adverse effects on muscle function. In addition, given that respiratory failure is the cause of death in most patients with DMD, it is

Previous articles in this series: No. 1: G.C. Sieck, Y.S. Prakash. Cross bridge kinetics in respiratory muscles. *Eur Respir J* 1997; 10: 2147–2158. **No. 2**: J.G. Gea. Myosin gene expression in the respiratory muscles. *Eur Respir J* 1997; 10: 2404–2410.

clear that dystrophin gene replacement will ultimately need to be targeted to the respiratory muscles in order to have a positive impact on patient survival.

Recombinant adenovirus vectors (AdVs): advantages and current limitations

AdVs are considered a promising means for deliver- ing a functional dystrophin gene to muscle. Advantages of AdVs as a vehicle for therapeutic gene transfer in DMD include the ability to infect nonreplicating muscle fibres, little apparent risk of oncogenesis, and the availability of replication-defective variants at high viral titres [8]. So-called "first generation" AdVs have been made replication-defective by a partial deletion of early (E) region 1 (E1) of the viral genome, which normally initiates viral gene transcription [9]. Elimination of the E3 region, which is involved in evading the host immune response [9, 10], allows for a further increase of exogenous gene insert size to about 7.5 kb for E1/E3-deleted AdVs. Although replication-defective AdVs hold great promise for delivering therapeutic genes to the diaphragm and other respiratory muscles, a major limitation to their usefulness has been the transient nature of transgene expression caused by immune-mediated destruction of the AdV-infected (transduced) cell population [11–15].

In this regard, immunological defense mechanisms that permit the host to combat viral infection can be broadly divided into two types: early nonspecific (innate) immunity and later specific acquired (adaptive) immunity [16]. In the early period of infection before effective acquired immune responses can be developed, natural killer (NK) cell activity can mediate cell lysis in a manner that does not show conventional antigen-specificity [16, 17]. Additionally, activation of antigen-presenting cells such as macrophages and release of cytokines such as tumour necrosis factor (TNF)-α, interferons (IFNs) and interleukins (ILs) from these and other cell types may also constitute early innate immune responses against viral infection [18]. Specific acquired immune responses that come into play later in the course of infection consist of both humoral (*e.g.*, neutralizing antibodies) and cell-mediated (*e.g.*, virus-specific cytotoxic T-lymphocytes (CTLs)) effector mechanisms [16]. Exogenous viral proteins endocytosed by antigen-presenting cells (*e.g.*, macrophages, dendritic and B cells) are presented as peptides by major histocompatibility complex (MHC) class II molecules to CD4+ T-cells; the CD4+ T-helper (Th2 subset) cell, in turn, is pivotal in generating a humoral response through release of cytokines (*e.g.*, ILs-4,5,10) that stimulate B cells [19, 20]. In contrast, endogenously synthesized viral proteins are presented by MHC class I molecules to CD8+ CTLs; once again, CD4+ T-helper (Th1 subset) cells release cytokines (*e.g.*, IL-2, IFN-γ, TNF-β) that are important in activating this response [19, 20]. Less frequently, CD4+ T-cells also appear to be capable of demonstrating direct CTL activity [16, 20].

In immunocompetent adult animals, studies in different target tissues have found that transgene expression is essentially eliminated 2–4 weeks after AdV delivery [11–15]. This was initially attributed to a CTL response against viral antigens presented to the surface of transduced cells by MHC class I molecules [11, 12], with consequent destruction of these cells. More recently, the transgene-encoded protein itself has been shown to be an important additional target of the CTL response [21]. Therefore, as a result of both adenoviral- and transgene-encoded foreign antigens, immunocompetent mice generate a prominent CTL response against transduced cells that peaks at 7–14 days and leads to their eventual elimination [11, 13, 14]. The problem is likely compounded by deletion of the E3 region from the vector, since the latter normally codes for genes involved in blocking transport of MHC class I antigens to the cell surface (*e.g.* E3-gp19K) as well as preventing TNF-induced cytolysis (*e.g.* E3-14.7K/E3-10.4K-14.5K) [10]. Genetically athymic (nude) mice, on the other hand, fail to exhibit lymphocytic infiltrates and maintain stable transgene expression for at least 2–3 months in lung and liver [11, 14]. Similarly, in adult nude [15] or severe combined immunodeficiency (SCID) mice [22, 23], long-term persistence of transgene expression can be attained after AdV injection into skeletal muscles, whereas immunocompetent mice demonstrate inflammatory cell infiltrates and complete elimination of the transgene [23].

The vast majority of studies utilizing AdVs in different organ systems, including muscle, have focused almost entirely on the level of transgene expression as their primary outcome measure. In contrast, there has been very little attention devoted to assessing the effects of AdV administration on target organ function. Following AdV administration to muscle, it is possible that toxic effects and/or immunological responses to AdVs could lead to impaired muscle contractility, thereby partially or even completely negating potential beneficial effects derived from therapeutic gene replacement. Therefore, to begin to address the problem of potential adverse effects on muscle function related to the vector itself, we have initially performed a series of experiments with AdVs encoding nontherapeutic marker genes in the mouse diaphragm. Additionally, these studies have been performed in immunocompetent normal and dystrophin-deficient mice with muscular dystrophy (mdx), as well as in other murine models with targeted immunological defects, in order to determine the influence of certain host cell factors upon the vector-host interaction.

Effects of AdV-mediated gene transfer to the diaphragm in normal and mdx mice

The diaphragms of normal control and mdx adult mice were directly injected (*via* laparotomy) with AdVs encoding one of two reporter genes: *Escherichia coli* LacZ, which encodes β-galactosidase (β-Gal); and firefly luciferase (Lux). These reporter gene products serve as sensitive marker proteins that allow quantification of the efficacy of AdV-mediated gene transfer and are not expected to directly affect muscle function. Figure 1 shows transverse cryosections of diaphragms from normal controls and mdx mice 1 week after AdV.LacZ administration. As can be seen, there is a fairly uniform distribution of β-Gal expression throughout the muscle cross-section in both groups of mice. In addition, the mean number and percentage of β-Gal positive fibres was significantly greater for the mdx mice group, as illustrated in figure 2. This is likely related, at least in part, to the high prevalence of regenerating muscle fibres in mdx mouse muscle, since previous work has indicated that immature muscle fibres

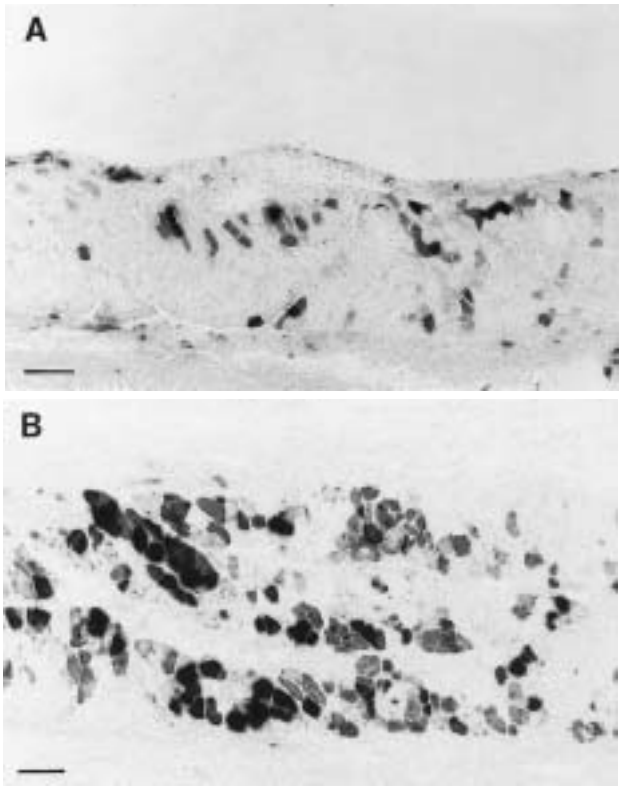

Fig. 1. – Transverse cryosections of diaphragms from: A) normal controls and B) dystrophin-deficient mice with muscular dystrophy (mdx mice). Muscle sections were stained for β-galactosidase activity (positive fibres are dark-staining) 1 week following direct *i.m.* injection of recombinant adenovirus vector(AdV).LacZ. Note the abnormal appearance of the mdx mice diaphragms (typical at this age), which are characterized by greater variability in fibre size, centrally nucleated fibres and scattered areas of necrosis with associated inflammatory cell infiltrates. Internal scale bar = 100 μm. Ref [24].

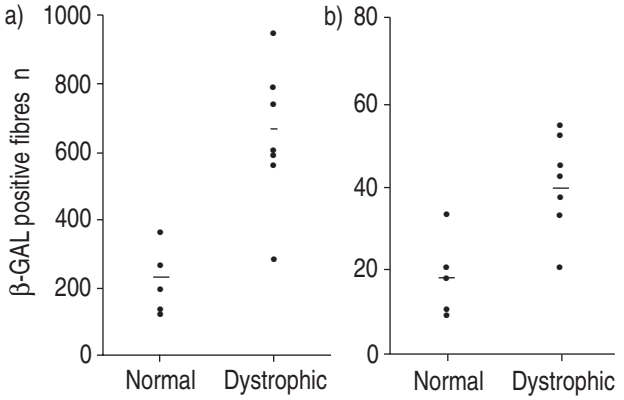

Fig. 2. – Transduction efficiency in the dystrophic diaphragm is superior to that in normal control diaphragm muscle one week post-injection of recombinant adenovirus vector(AdV).LacZ. a) Total number of fibres expressing β-galactosidase (β-Gal) in normal control and dystrophic diaphragms. b) Percentage of β-Gal positive fibres in normal and dystrophic diaphragms. Values are presented as mean±SEM. Both the absolute number and relative percentage of β-Gal positive fibres were significantly higher in the dystrophic group. Ref [24].

are more susceptible than adult fibres to AdV-mediated gene transfer [25–27]. One potential explanation for the greater transducibility of immature muscle fibres is the higher level of αv chain containing integrins [27], which form part of the adenovirus internalization receptor [28].

As can be seen in figure 1, the mdx mice diaphragms also showed the typical features of highly variable fibre size, centrally nucleated fibres and inflammatory cell infiltrates, all of which are normally present in this muscle [29, 30]. However, control mouse diaphragms injected with AdV.LacZ also showed small foci of inflammation in some areas, and this was not observed in sham phosphate-buffered saline (PBS)-injected diaphragms from these mice. At later time points (10–20 days), even greater degrees of inflammatory cell infiltration were observed in control mouse diaphragms injected with AdV.LacZ, which appeared to be specifically targeting β-Gal positive fibres in many instances. Using immunohistochemical methods, we have identified these cells as consisting primarily of CD8+ and CD4+ lymphocytes [31]. By 1 month post-AdV administration, this immunological attack against AdV-infected fibres resulted in virtually complete elimination of transgene expression in mdx as well as control mouse diaphragms [24, 31]. Furthermore, as shown in figure 3, a major decrease in maximal tetanic force generation was found 1 month after AdV injection in both normal and mdx mice as compared to their sham-injected counterparts.

We also tested the hypothesis that immunosuppressive treatment with cyclosporine, by inhibiting T-lymphocyte activation, would abrogate adverse effects on transgene expression and diaphragm function caused by cellular immune responses. Interestingly, although immunosuppressive therapy with cyclosporine had no impact on the level of transgene expression in the diaphragm, a significant effect on contractile function of the muscle was nonetheless observed. Thus, the reduction in force production after one month was significantly alleviated in the cyclosporine groups for normal as well as mdx mice (fig. 3). Because the lack of effect on transgene expression indicates that cyclosporine was unable to prevent immune-mediated elimination of transduced fibres, the higher force-generating capacity of immunosuppressed animals implies that cyclosporine probably improved muscle function by limiting

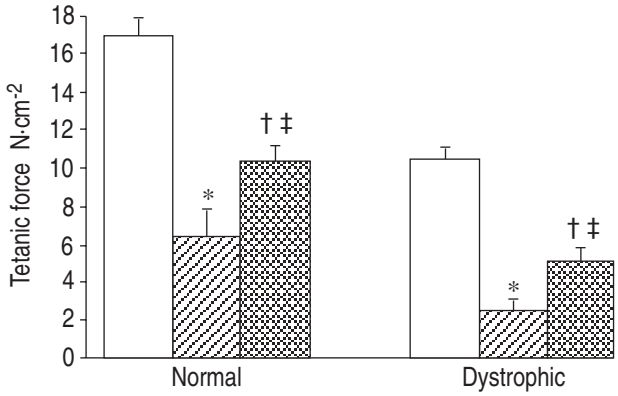

Fig. 3. – Maximal force-generating capacity of normal control and dystrophic diaphragms examined 30 days after recombinant adenovirus vector (AdV) administration. In both groups of mice, there was a significant reduction in force production compared to sham phosphate-buffered saline (PBS)-injected counterparts. This AdV-related decrease in force-generating capacity was partially reversed by cyclosporine treatment. Values are presented as mean±SEM. ▭: PBS-injected diaphragm (sham); ▨ : AdV-injected diaphragms + daily *i.p.* injection of saline (AV-saline); ▩ : AdV-injected diaphragms + daily *i.p.* injection of cyclosporine at a dose of 15 $mg \cdot kg^{-1} \cdot day^{-1}$ (AV-cyclosporine). *: $p < 0.01$, sham *versus* AV-saline; †: $p<0.01$, sham *versus* AV-cyclosporine; ‡: $p<0.05$, AV-saline *versus* AV-cyclosporine. Ref [24].

the degree of damage to the surrounding population of nontransduced muscle fibres. In other words, our data suggest that following AdV injection of muscle, the immune system causes damage to nontransduced fibres ("bystan-der effect") as well as transduced cells. One possible ex-planation for such an effect on nontransduced fibres would be the release of various cytokines, which have previously been associated with impaired diaphragm contractility *in vitro* [32]. In addition, we have recently reported evidence for increased expression of inducible nitric oxide synthase (iNOS) within macrophages infiltrating mdx mice muscles in the early post-AdV delivery period [33]. Since nitric oxide (NO) produced by endothelial cells depresses con- tractility of adjacent cardiomyocytes [34] and also reduces diaphragm force-generating capacity [35, 36] *in vitro*, NO could conceivably act in concert with cytokines to pro- duce adverse effects on the contractile function of nontransduced as well as transduced muscle fibres.

Interference with T-lymphocyte subsets enhances transgene expression and force-generating capacity after AdV delivery to the diaphragm

Because the above experiments suggested an important role for T-lymphocytes in the impairment of force-generating capacity observed after AdV injection [24], we were interested in determining the effects of interfering with either CD4+ or CD8+ T-cell subsets [31]. To study the former, we administered GK1.5, a neutralizing anti-CD4 monoclonal antibody (CD4Ab); to assess the latter, we employed β_2-microglobulin knockout (β_2m-) mice, which are lacking in effective CD8+ T-lymphocyte function [37]. Results of β-Gal transgene expression in four experimental groups are depicted in figure 4. As shown earlier, β-Gal expression was largely eliminated by 30 days in the immunocompetent normal control group. However, these animals demonstrated significantly higher levels of transgene expression with the addition of CD4Ab treatment. In addition, the β_2m- group lacking CD8+ T-cells showed a persistent high level of β-Gal expression at 30 days that did not differ significantly from that seen at 4 days post-injection (data not shown). Furthermore, there was no significant effect of CD4Ab administration on β-Gal expression in β_2m- animals, although a trend towards greater β-Gal expression was noted.

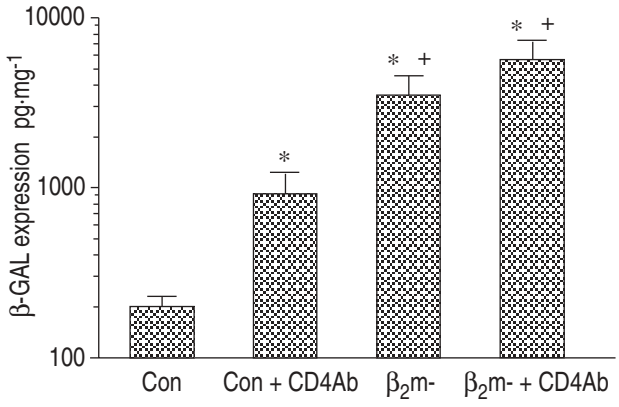

Fig. 4. – Transgene expression in the diaphragm 30 days after recombinant adenovirus vector (AdV).LacZ administration is enhanced by interference with either CD4+ or CD8+ T-cell function. In comparison to immunocompetent control (Con) mice, there was significantly greater β-Gal expression after 30 days in otherwise immunocompetent animals receiving systemic administration of anti-CD4 monoclonal antibody (Con + CD4Ab). In addition, β-Gal expression was also significantly higher in the two groups of β_2-microglobulin knockout (β_2m-) mice, both in the presence and absence of superimposed CD4Ab administration. Values are presented as mean±SEM. *: $p<0.05$ *versus* Con; +: $p<0.05$ *versus* Con + CD4Ab [31].

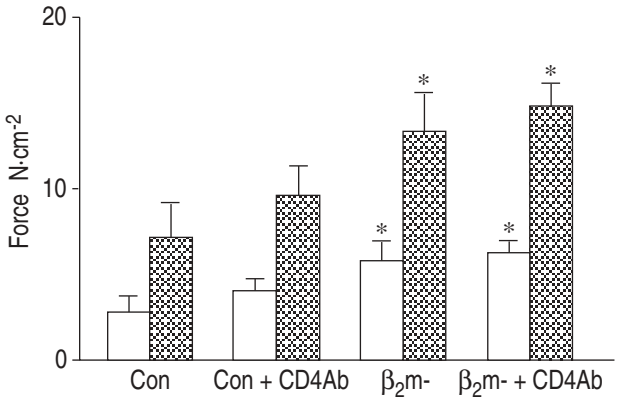

Fig. 5. – Effects of interference with CD4+ and CD8+ T-cell function on maximal twitch (Pt) and maximal tetanic (Po) force generation by the diaphragm 30 days after recombinant adenovirus vector.LacZ administration. Values are presented as mean±SEM. ▭ : Pt; ▩ : Po. Although there was a trend towards higher force production after anti-CD4 monoclonal antibody (CD4Ab) treatment, this did not achieve statistical significance. However, β_2-microglobulin knockout (β_2m-) mice lacking effective CD8+ T-cell function demonstrated significantly increased force-generating capacity as compared to immunocompetent control (Con) mice. *: $p<0.05$ *versus* Con [31].

The effects of inhibiting host CD4+ and CD8+ T-cell function on muscle force production after AdV.LacZ administration are shown in figure 5. In keeping with our previous findings [24], maximal twitch (Pt) and tetanic force (Po) production by the diaphragm at 30 days post-AdV injection were substantially depressed in the control group. On the other hand, in β_2m- animals lacking CD8+ T-cells, diaphragm force-generating capacity was significantly increased as compared to the control mice. In addition, both control and β_2m- mice demonstrated a trend towards greater force production with the addition of CD4 Ab treatment. Therefore, these data indicate that CD8+ T-lymphocytes play the major role in eliminating transgene expression as well as causing destructive immune responses that lead to impaired diaphragm force-generating capacity after AdV-mediated gene transfer. Although less important, CD4+ T-cells also appear to be involved in these adverse effects, which could be due to direct CD4+ CTL-mediated actions [16, 20] and/or CD4+ T-helper cell-mediated amplification of the CD8+ CTL response [38, 39].

Nonimmune muscle fibre toxicity is linked to AdV transcriptional activity

Although interference with T-lymphocyte function was able to significantly alleviate the decrease in diaphragm force-generating capacity observed after AdV injection, maximal force production remained 20–30% below that of sham (PBS)-injected diaphragms. Therefore, we hypothesized that this residual loss of muscle force could be due to a direct toxic effect of AdV on muscle fibres that was independent of the cellular immune response. In

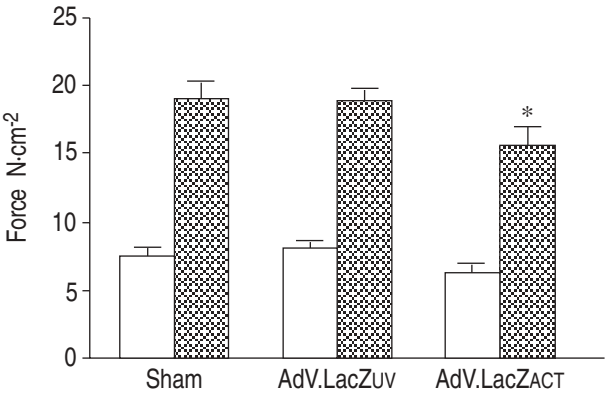

Fig. 6. – Effect of inactivating AdV gene transcription on maximal twitch (Pt) and maximal tetanic (Po) force-generation of the diaphragm. □ : Pt; ▨ : Po. Values are presented as mean±SEM. Injection of transcriptionally active recombinant adenovirus vector(AdV.LacZACT) in β_2-microglobulin knockout mice led to a significant decrease in Po as compared to sham (PBS)-injected counterparts. In contrast, force production in diaphragms injected with ultraviolet (UV)-inactivated AdV (AdV.LacZUV) did not differ from sham-injected animals. *: $p<0.05$ *versus* sham. Ref [31].

particular, we wondered whether low-level expression of adenoviral gene products *per se* might be involved in myofibre cytotoxicity. For instance, the E4 region of the adenoviral genome encodes gene products with a number of functions including blockage of both host cell protein synthesis [40] and transcriptional activation of cellular growth proteins by the p53 tumour suppressor [41]. In order to begin to address this issue, we have compared the effects of transcriptionally active and ultraviolet (UV)-inactivated AdV particles on diaphragm contractility. AdV.LacZ was pretreated with 8-methoxypsoralen and exposed to a 365 nm light source as previously described [42, 43]. This procedure has been shown to eliminate viral gene transcription while preserving AdV particle entry into target cells and endosomolytic activity [42]. The same viral titre was then used for *in vivo* injection of both UV-inactivated (AdV.LacZUV) and active (AdV.LacZACT) preparations. To eliminate effects related to cellular immunity, these experiments were performed during the early post-AdV delivery period (prior to the development of substantial lymphocyte invasion) and in β_2m- mice lacking the capacity to generate an effective CD8+ CTL response.

Figure 6 shows the effects of eliminating AdV-related gene expression on muscle force production 4 days after AdV delivery to the diaphragms of β_2m- mice. The average reduction in maximum Po force generation after AdV.LacZACT injection (compared to sham counterparts) amounted to approximately 20%. Force-generating capacity was significantly greater in animals receiving AdV.LacUV than in β_2m- mice injected with AdV.LacZACT. Furthermore, maximal force production in the AdV.LacUV group did not differ from sham (PBS)-injected β_2m- animals. Therefore, the data are consistent with the presence of a significant inhibitory effect on diaphragm force production by the presence of a functional AdV genome, even in the absence of effective T-lymphocyte responses.

Conclusions

Our data indicate that AdV-mediated transfer of reporter genes (as opposed to the therapeutic dystrophin gene) to diaphragm muscle causes significant impairment of force generation in normal as well as mdx mice. Experiments employing immunodeficient animal models point to two major aetiologies for this reduction in force-generating capacity: 1) a nonantigen-specific myofibre toxicity of early onset that is linked to AdV transcriptional activity; and 2) an antigen-specific immune-based component of more delayed onset that is dependent upon intact CD8+ T-cell activity. These findings raise the concern that toxic effects and immunological responses to AdV administration could partially or even completely negate the therapeutic effects of dystrophin gene transfer to the diaphragm in DMD patients. A number of potential strategies for overcoming these problems are suggested by this investigation. First, manipulations of the AdV genome such as additional major deletions of viral genes [44, 45] and the use of temperature-sensitive AdV mutations [13, 14] may be useful in reducing the impairment of muscle contractility associated with a transcriptionally active viral genome. Second, attempts to blunt T-cell- mediated immunity against AdV-infected fibres through the use of less immunogenic vectors, perhaps in combination with immunosuppressive antibodies [31, 46] and/or drugs [33], will also be important. Experiments are currently in progress to explore the ability of these strategies to improve dystrophic diaphragm function after AdV-mediated transfer of the therapeutic dystrophin gene.

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