Immunological hurdles in the path to gene therapy for Duchenne muscular dystrophy

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Patients with Duchenne muscular dystrophy (DMD), an X-linked lethal muscle-wasting disease, have abnormal expression of the protein dystrophin within their muscle fibres. In the *mdx* mouse model of this condition, both germline and neonatal somatic gene transfers of dystrophin cDNAs have demonstrated the potential of gene therapy in treating DMD. However, in many DMD patients, there appears to be no dystrophin expression when muscle biopsies are immunostained or western blots are performed. This raises the possibility that the expression of dystrophin following gene transfer might trigger a destructive immune response against this 'neoantigen'. Immune responses can also be generated against the gene transfer vector used to transfect the dystrophic muscle, and the combined immune response could further damage the already inflamed muscle. These problems are now beginning to be investigated in immunocompetent *mdx* mice. Although much work remains to be done, there are promising indications that these immune responses might not prove as much of a concern as originally envisaged.

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Duchenne muscular dystrophy (DMD) is an X-linked recessive disease characterised by repeated rounds of necrosis and regeneration of the skeletal muscles, leading to fibrosis and muscle weakness. The incidence of the disease is approximately 1 in 3500 live male births (Ref. 1). Affected boys are usually diagnosed at 3–4 years old when problems with walking become noticeable. Patients are usually confined to a wheelchair by the age of 11 years, and death from respiratory or cardiac failure is common in the late teens to early 20s. With improved nursing care and positive pressure ventilation, some patients are now reaching their 30s (Ref. 2).

Following the discovery that DMD is caused by mutations in an exceptionally large gene (3 Mb) encoding dystrophin (Ref. 3), there has been a great impetus to achieve a cure for this disease. Various strategies have been devised to re-establish dystrophin expression in deficient muscle fibres and these are briefly reviewed below. However, the de novo expression of dystrophin in affected tissues carries its own risk – that of eliciting an immune response that might result in further damage to muscle fibres. This article focuses particularly on this problem, reviewing evidence from studies to date on immune responses to dystrophin and discussing the clinical significance of these findings.

Dystrophin and DMD

Dystrophin is expressed in all skeletal muscles as a large (427 kDa) protein (Fig. 1a). It is located at the plasma membrane (or sarcolemma) of skeletal and cardiac muscle fibres and is absent or abnormal in biopsies from muscles of DMD patients (Ref. 3; Fig. 2). Over the past decade, a variety of proteins have been shown to be associated with dystrophin (Fig. 1b) and in turn have themselves been associated with other, clinically similar muscular dystrophies (reviewed in Ref. 4).

Many different mutations have been discovered in DMD patients, ranging from point mutations to the more common large deletions and insertions (for details see http://www.dmd.nl/md.html) Such mutations result in mRNA instability, and/or bring about premature termination of translation leading to an unstable incomplete protein that is nonfunctional and rapidly degraded. Some mutations in the dystrophin gene lead to deletion of part of the coding sequence but maintain the reading frame; such mutations result in a semifunctional, internally deleted, dystrophin protein and result in a milder (Becker) muscular dystrophy (BMD) (Ref. 5).

The dystrophin gene also produces multiple other dystrophin isoforms, which arise from a variety of tissue-specific promoters and alternative splicing events. Alternative splicing of the first exon and expression from the cortical promoter or the Purkinje promoter produces the full-length (427 kDa) brain isoforms (Refs 6, 7). Other promoters exist within the introns further downstream. These promoters give rise to tissue-specific truncated isoforms such as Dp45 and Dp71 (expressed in all tissues except muscle), Dp116 (expressed in Schwann cells), Dp140 (expressed in central nervous tissue and kidney) and Dp260 (expressed in retina). The different isoforms are depicted in Figure 3 and reviewed in Ref. 8, and further information can be found at the Leiden University website (http://www.dmd.nl/). An additional promoter 600 kb upstream of all the other promoters drives expression in lymphocytes, extending the gene to 3 Mb in length (Ref. 9). Further complexity comes from the numerous alternative splice variants found at the C-terminus of the muscle isoform. At least 18 splice variants have been described for this region (reviewed in Ref. 10). Thus, the site of DMD mutation will determine which isoforms (if any) are expressed in each DMD patient.

Developing therapies for DMD

Although genetic counselling can reduce the prevalence of the condition within a family, approximately a third of all cases are the result of spontaneous new mutations. There is a pressing need to develop treatments for this condition and several therapeutic options are being explored (Table 1). The development of such treatments has been greatly assisted by the discovery of a natural genetic homologue of DMD: the *mdx* mouse (Ref. 11). The *mdx* mouse is dystrophin deficient owing to a point mutation that leads to a premature stop codon (i.e. a nonsense mutation) and the production of an unstable peptide (Ref. 12). Transgenic *mdx* mice expressing recombinant dystrophin cDNAs have revealed a wide therapeutic window, with prevention of pathology being achieved with dystrophin expression ranging from 0.2-times to 50-times normal endogenous levels (Refs 13, 14, 15).

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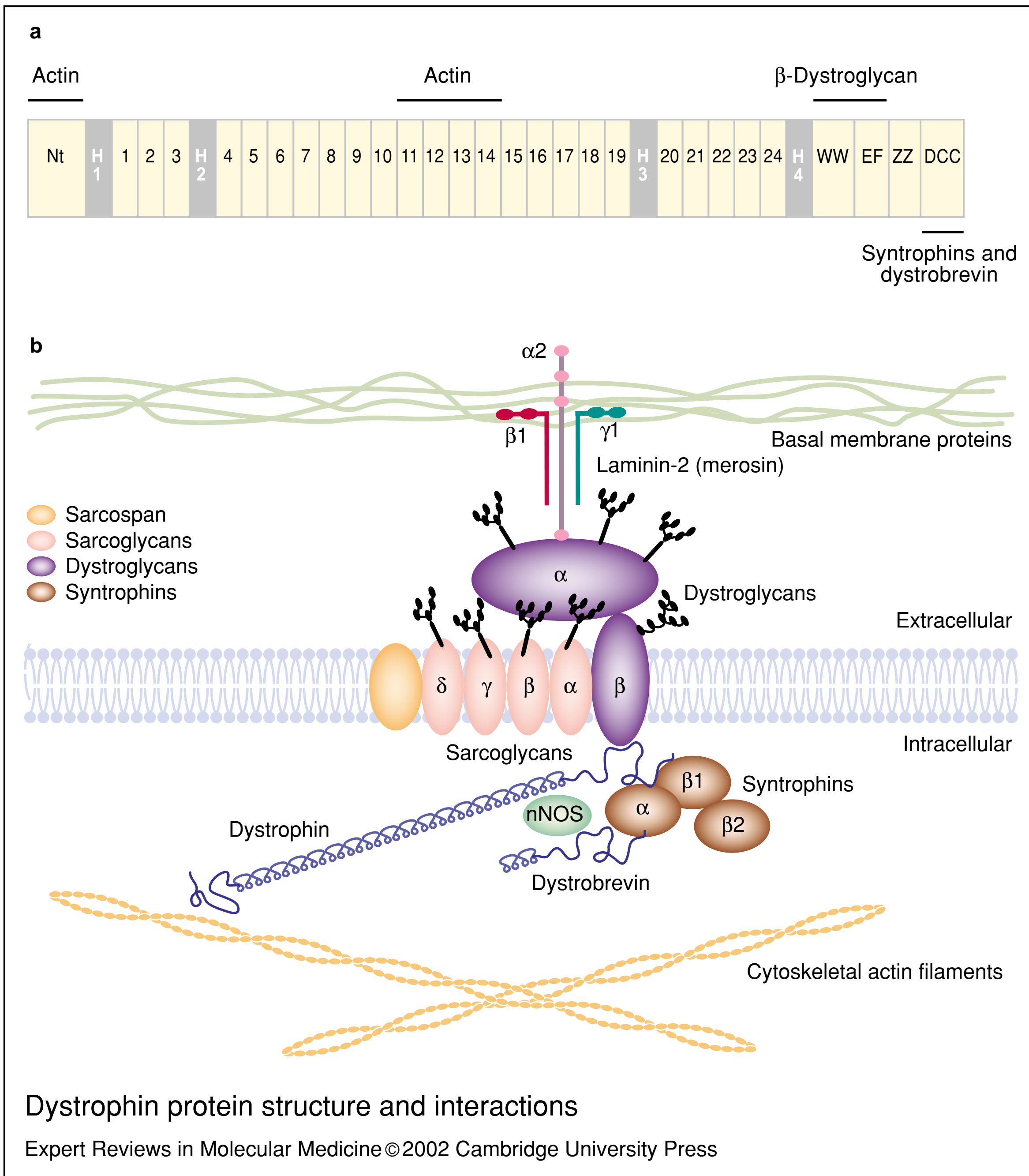

Figure 1. Dystrophin protein structure and interactions. (a) Diagram of dystrophin showing the main domains: N-terminus (Nt); rod domain of 24 triple-helical coiled-coil repeats and four hinges (H1–4); and the subdomains of the C-terminus (WW, WW domain; EF, EF hand calcium-binding domain; ZZ, ZZ zinc finger domain; DCC, predicted dimeric coiled-coil domain). The locations of the binding sites for actin, β-dystroglycan, syntrophins and dystrobrevin are indicated. (b) Diagram of the localisation of dystrophin and the dystrophin-associated proteins. Dystrophin forms a link between the actin cytoskeleton and the extracellular matrix via the dystroglycans. Sarcospan and the sarcoglycans stabilise the membrane complex. An intracellular complex including the syntrophins and dystrobrevin together with dystrophin stabilises the signalling molecule neuronal nitric oxide synthase (nNOS) in a subsarcolemmal position. Thus, dystrophin and the associated proteins are thought to have both a structural and a signalling role in the muscle fibre. Loss of this complex as a result of mutations in dystrophin or one of the associated proteins leads to muscular dystrophy **(fig001dwl)**.

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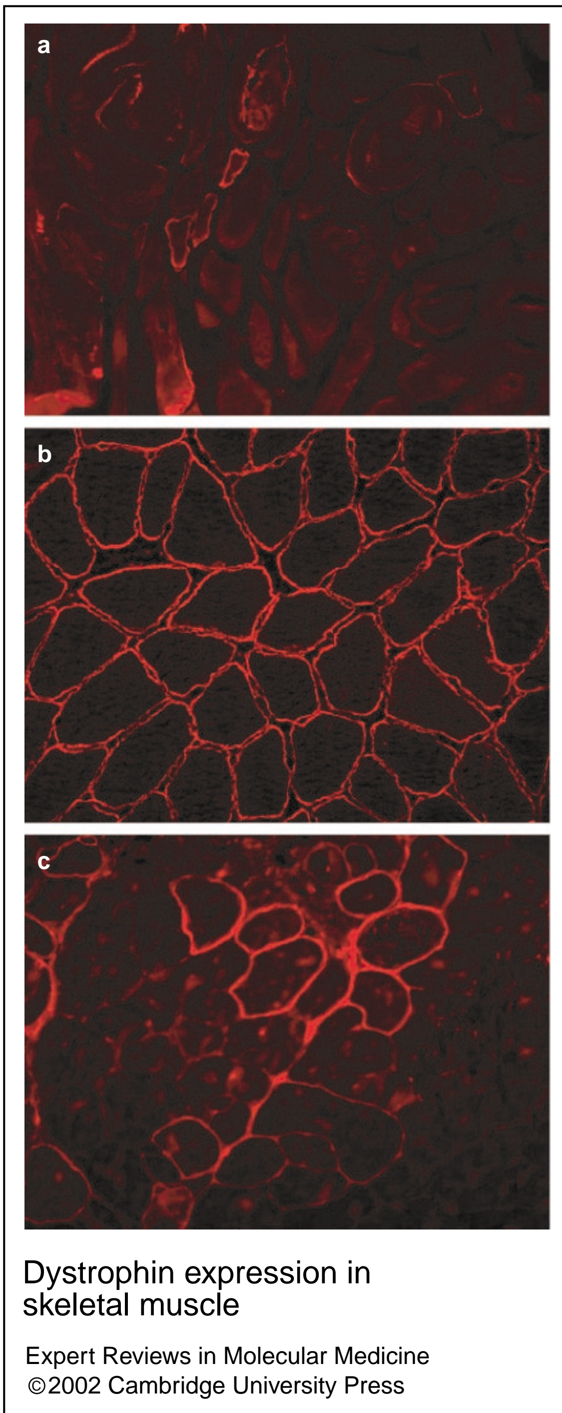

Figure 2. Dystrophin expression in skeletal muscle. Immunofluorescent photomicrographs of cross-sections of skeletal muscle stained with antibodies recognising dystrophin: (a) Duchenne muscular dystrophy (DMD) muscle; (b) normal muscle; (c) muscle from the *mdx* mouse model, showing partial correction of dystrophin deficiency following plasmid-based gene transfer of a full-length dystrophin cDNA. Dystrophin appears as a sarcolemmal staining in normal muscle that is absent in DMD and *mdx* fibres but is restored following gene transfer. Images in (a) and (b) are courtesy of Dr Sue Brown, Dubowitz Neuromuscular Centre, Imperial College, Hammersmith Hospital, London, UK. The width of each image is approximately 250 µm **(fig002dwl)**.

Dystrophin expression can be restored in *mdx* mice by several routes, including drug administration, the use of oligonucleotides, cell transplantation, and dystrophin gene transfer with viral or nonviral vectors. Some of these studies, together with attempts to transfer these strategies to patients, are discussed below.

Repair/modification of mutated gene

Barton-Davis and colleagues (Ref. 16) reported that high doses of the aminoglycoside antibiotic gentamicin could lead to substantial expression of dystrophin by the read-through of the stop mutation in *mdx* mice. This approach could have application in up to 15% of DMD patients (those with nonsense mutations) but the results of preliminary clinical trials have not been promising (Ref. 17).

Chimaeric RNA/DNA oligonucleotides ('chimaeroplasts') can be used to induce single-base-pair changes in the host genome (gene repair). The RNA moiety leads to enhanced binding of the oligonucleotide to the specific DNA region and encourages mismatch repair (a normal nuclear activity to correct DNA damage). Using this technique, Rando and co-workers (Ref. 18) demonstrated gene repair in a small number of *mdx* muscle fibres following intramuscular administration. Similar results have been reported for the canine Golden Retriever muscular dystrophy (GRMD) model of DMD (Ref. 19). If successful in humans, this approach could be used to treat about 20% of DMD patients (those with point mutations).

Modification of transcript of mutated gene

An alternative approach to gene repair/modification is to encourage 'exon skipping', a process that is believed to be responsible for the presence of dystrophin-positive 'revertant' fibres in humans and animals that otherwise have dystrophin deficiencies (Refs 20, 21). Exon skipping is thought to occur in the nucleus at the time of splicing of the primary transcript to form

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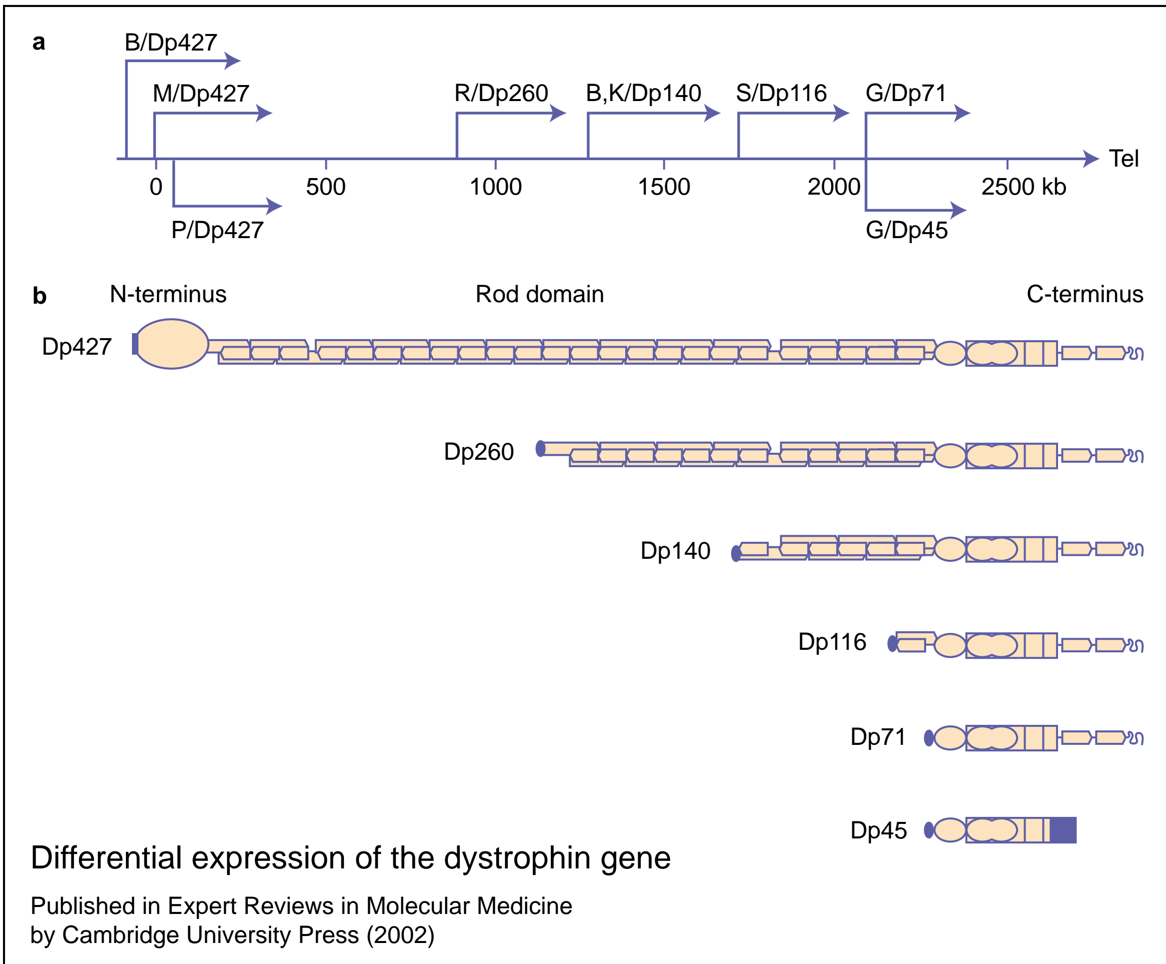

Figure 3. Differential expression of the dystrophin gene. (a) Diagram of the dystrophin gene showing the position of the different promoters (B, brain; M, muscle; P, Purkinje; R, retina; B,K, brain and kidney; S, Schwann cells; G, general). (b) Diagram of the dystrophin isoforms, comparing the main domains of full-length Dp427 with those of Dp260, 140, 116, 71 and 45. Only Dp427 contains the N-terminal actin-binding domain, but most of the other isoforms contain some triple-helical coiled-coil repeats (repeats indicated by the parallel rods; hinges indicated as notches), and the subdomains of the C-terminus [single circle, WW subdomain; double circle, EF subdomain; double boxes, ZZ subdomain; two bars, DCC subdomain (see Fig. 1)]. Figure reproduced from Ref. 10, with kind permission of Kluwer Academic Publishers **(fig003dwl)**.

mRNA. Thus, by favouring abberant splicing of noncontiguous exon regions, the effect of the nonsense mutation is overcome and a dystrophin protein with sufficient functional domains to mediate the plasmalemmal assembly of the dystrophin-associated glycoprotein complex is produced. Several studies have shown that exon skipping can also be induced with oligonucleotides in *mdx* cells in culture (Refs 22, 23) and in lymphocytes and myoblasts from DMD patients (Refs 24, 25, 26). Wilton and colleagues have recently demonstrated successful induction of exon skipping in vivo after multiple injections of oligonucleotide (Ref. 27). This approach is promising as it can be adapted to the patient's genotype (i.e. the precise location of the mutation) to encourage the formation of an in-frame transcript, and thus could be applicable to the vast majority of patients. Although proteins arising from this approach will be partially deleted, transgenic mouse studies suggest that such proteins might have some functional benefit (reviewed in Ref. 28). In the studies cited above, experiments were conducted in vitro or involved direct injection into muscle in vivo. However, as this approach

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Table 1. A selection of possible approaches for the treatment of Duchenne muscular dystrophy (tab001dwl)

Treatment	Effect	Species	Ref.
Corticosteroids	Reduces speed of progression of disease	Human, mouse	108
Aminoglycosides (gentamicin)	'Read-through' of stop mutations to produce dystrophin	Mouse	16
Gene upregulation (utrophin)	Utrophin is expressed normally in embryonic muscle; overexpression in mature muscle compensates for dystrophin in transgenics	Mouse	109
Protease inhibitor (leupeptin)	Specific inhibition of muscle-specific isoform of calpain, the major protease in muscle, reduces loss of muscle fibres	Mouse	55
Metabolic supplements	Variety of compounds improve strength in *mdx* mice, but might be less successful in humans	Human, mouse	110
Gene repair (chimaeroplasts)	Mismatch repair of point mutations in mutated dystrophin gene	Mouse	18
Exon-skipping (spliceomers)	Modification of splicing of primary transcript to restore reading frame and protein production	Mouse	27
Cell transplantation	Introduction of functional dystrophin through donor muscle precursor cells or genetically modified self non-muscle cells	Human, mouse	111 (review)
Viral-based gene transfer	Transfer of dystrophin cDNA directly into muscle using viral vectors	Mouse, dog	28 (review)
Nonviral-based gene transfer	Transfer of dystrophin cDNA directly into muscle using nonviral (plasmid DNA) vectors	Mouse, dog	28 (review)

modifies only mRNA translation, regular re-administration will be required. Patients are unlikely to tolerate repeated injections into multiple muscles. A better solution would be to deliver the treatment systemically via the blood supply. There is a substantial literature regarding the systemic delivery of oligonucleotides to many tissues, usually as a complex with lipids to increase stability, although there is little about delivery to muscle. Some possible ligands for targeting oligo–lipid complexes to muscle have been reviewed by Feero and colleagues (Ref. 29).

Cell transplantation

Cell transplantation with donor muscle precursor cells offers not only the introduction of a functional dystrophin gene into dystrophic muscle but also the replenishment of the myoblast pool for the repair of damaged fibres. However, this approach is limited by the local nature of cell delivery and by immune responses to donor cells. Genetic modification of the DMD patient's myoblasts is not possible as these cells are bordering on senescence when collected (Ref. 30). Several research groups are investigating the potential for genetic modification of other cell types that can be collected from the patient (Refs 31, 32). Bone marrow is a promising candidate as experiments in *mdx* mice suggest that bone-marrow-derived cells can contribute to muscle repair (Ref. 33). However, more-recent work has shown that this process is very inefficient and it has not been possible to increase efficiency substantially (Ref. 34).

Gene therapy

Since the discovery of dystrophin in 1987, a large number of laboratories have been working on the delivery of dystrophin cDNAs to skeletal muscle (reviewed in Ref. 28). Such studies were aided by

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the description of an internally deleted dystrophin cDNA derived from a patient with very mild BMD (Ref. 35). The full coding sequence for dystrophin is 11.5 kb, which was too large for most of the viral vectors that were available in the early 1990s. However, the gene isolated from the BMD patient was deleted for most of the internal rod domain of the molecule, resulting in a coding sequence of just over 6 kb, and so could be conveniently cloned into the early generation of viral vectors. This recombinant dystrophin with the internal deletion is commonly referred to as the minidystrophin construct. A variety of viral vector systems carrying recombinant dystrophins have been tested in *mdx* mice, including vectors based on adenovirus, retrovirus, herpesvirus and adeno-associated virus (Ref. 28).

An alternative to the use of viral vectors for gene delivery is to deliver genetic material in the form of bacterial plasmid DNA. In the simplest form, naked plasmid DNA can be injected into skeletal muscle, leading to transfection of muscle fibres close to the site of delivery (Ref. 36). Indeed, this was the first method used to transfer and express dystrophin constructs in the *mdx* mouse (Ref. 37). Surprisingly, the addition of lipids and or ligands to the plasmid DNA, a process that substantially increases transfection efficiency for cells in culture, actually decreases the efficiency of gene transfer into skeletal muscle in vivo (D.J. Wells, unpublished).

The major problem associated with plasmid-based gene delivery to skeletal muscle is the relatively low efficiency of transfection. However, it has been possible to show that dystrophin plasmid gene transfer into skeletal muscle provides protection from fibre turnover in the *mdx* mouse as measured by the maintenance of luciferase expression from a co-injected plasmid (Ref. 38). The diaphragm can also be transfected by direct plasmid injection; the tightly confined space between the fascia provides good exposure of muscle fibres to the DNA solution (Refs 39, 40). Recent developments have demonstrated improvements in efficiency. Regional intra-arterial delivery to the limbs of rats results in a greater level of transfection than does local injection (Ref. 41) and this work has recently been extended to studies in primates (Ref. 42). No adverse effects of this pressure-based delivery have been observed other than transient swelling of the limb. Local delivery can also be enhanced by the application of an electrical field to the muscle after injection of the plasmid DNA (Refs 43, 44, 45, 46). Pre-treatment of the muscle with enzymes such as hyaluronidase enhances the electroporation effect, thus allowing a decrease in the field strength and consequently a reduction in the myofibre damage associated with electroporation (Ref. 47). This raises the efficiency of nonviral gene delivery to that achievable with local delivery using the best viral vector.

Potential for immune responses to dystrophin

In all of the above examples, the aim has been to produce dystrophin in previously dystrophin-deficient muscle. However, a serious concern is the possibility that dystrophin will be perceived by the immune system as a neoantigen and that, consequently, dystrophin expression might be lost by the destruction of the modified muscle fibres. Indeed, if this is the case, the act of restoring dystrophin expression could lead to an increased loss of muscle fibres in patients that have already lost many of their muscle fibres through the dystrophic process. The current evidence for immune responses to newly expressed dystrophin and the vector itself is discussed here in more detail.

Antigen presentation following gene transfer into muscle

The immunobiology of muscle and the immunological consequences of plasmid gene transfer into muscle have both been reviewed recently (Refs 48, 49). The following is a brief summary of the most important elements.

Antigen presentation underlies the generation of a specific immune response to foreign proteins. In order for T cells to recognise and respond to an antigen, the antigenic peptide must be processed and presented on the surface of an antigen-presenting cell (APC), such as a dendritic cell (DC), in the context of major histocompatibility complex (MHC) molecules. APCs can be directly transfected following gene transfer into muscle or can endocytose the gene product. They then migrate to local lymphoid tissue where they present potential antigens in the context of the MHC molecules to T cells. MHC class I molecules are used to present antigens to $CD8^+$ T cells, whereas MHC class II present antigens to $CD4^+$ T cells (Fig. 4). Presentation of antigen to $CD4^+$ T cells causes activation and, depending on the antigen and the local cytokine environment, these

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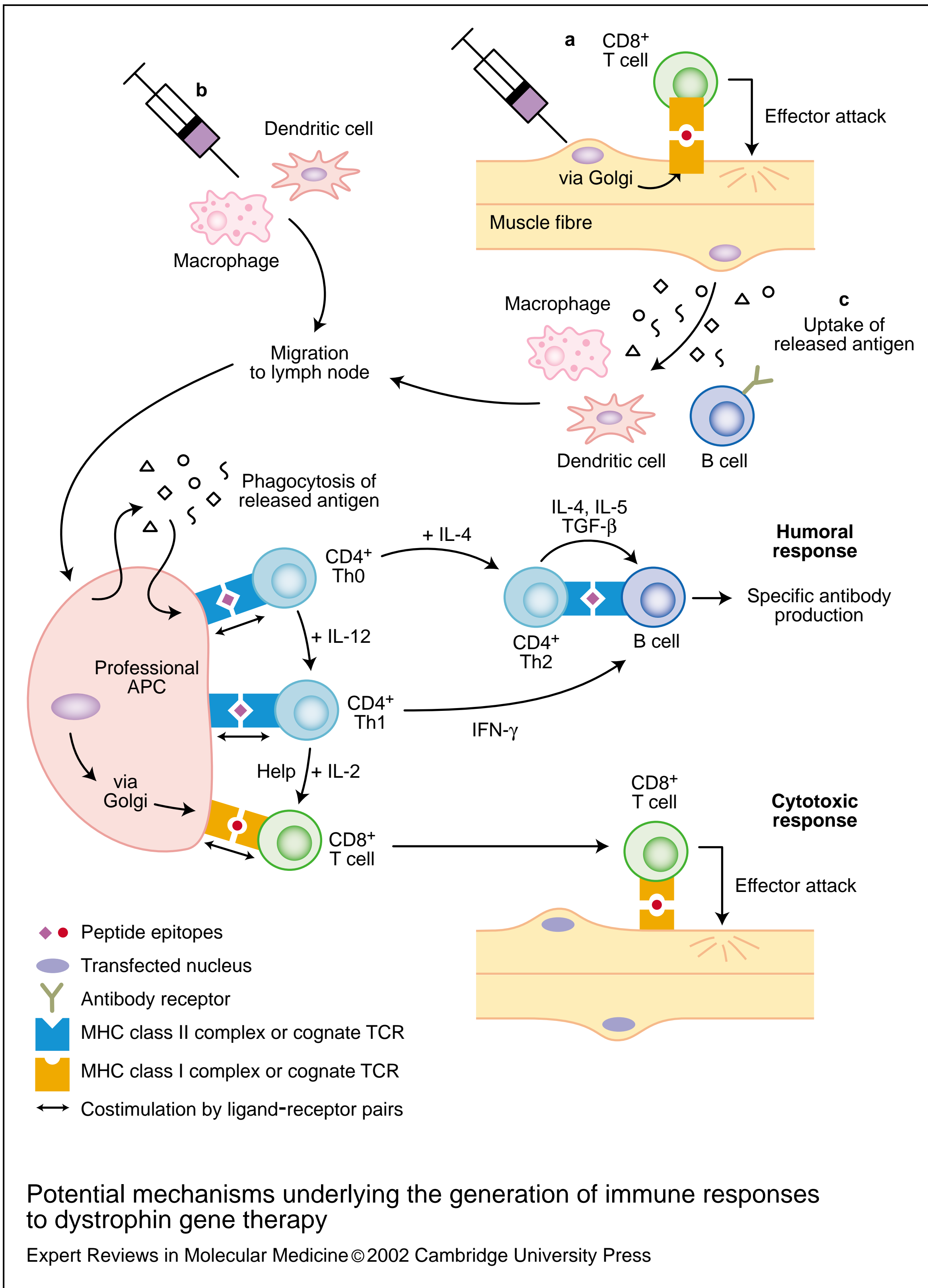

Figure 4. Potential mechanisms underlying the generation of immune responses to dystrophin gene therapy *(see next page for legend)* **(fig004dwl).**

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Figure 4. Potential mechanisms underlying the generation of immune responses to dystrophin gene therapy. The diagram shows possible systems for the presentation of dystrophin to cells of the immune system following its expression as a result of gene transfer to dystrophic muscle, and subsequent generation of humoral and cytotoxic responses. (a) Skeletal muscle might act as a direct antigen-presenting cell (APC), but this is unlikely as it lacks costimulatory molecules. (b) Professional APCs (macrophages and dendritic cells) might be directly transfected by certain vectors. (c) Classically, external antigens are taken up and processed for presentation via major histocompatibility complex (MHC) class II, and antigens produced within the APC are processed for presentation via MHC class I. However, in 'cross-priming' dystrophin released from transfected muscle is taken up by professional APCs and processed for presentation via both MHC class I and class II (in the absence of direct expression in the APCs). Macrophages tend to take up particulate antigens, dendritic cells take up soluble antigens and B cells can act as APCs if the antigen can bind to their specific antibody receptor. Following migration of APCs to the lymph node, antigens are presented to $CD4^+$ T cells in the context of MHC class II and to $CD8^+$ T cells via MHC class I. Depending on the cytokine environment, $CD4^+$ T cells differentiate as either Th1 (T helper 1) or Th2 cells. Th1 cells help activate $CD8^+$ T cells to respond to antigens presented via MHC class I and this forms the cytotoxic arm of the acquired immune response. Th2 cells help activate B cells to respond to antigens, forming the humoral arm of the acquired immune response. Secretion of interferon γ (IFN-γ) by Th1 cells can switch the isotype of antibodies produced by the B cell from IgG_1 to predominantly IgG_{2a}. Thus, the latter isotype serves as an indirect indicator of a cytotoxic immune response. IL, interleukin; TGF, transforming growth factor **(fig004dwl)**.

T cells can become either Th2 cells and activate humoral responses of B cells or Th1 cells and stimulate $CD8^+$ T cells. Antigen presentation in the context of MHC class I together with the $CD4^+$ Th1 help generates activated $CD8^+$ cytotoxic T cells that can destroy target cells expressing the same MHC-class-I-bound antigen. Normal muscle fibres do not express detectable levels of MHC class I or class II, but levels of both are increased in inflamed muscle. APCs also express adhesion and costimulatory molecules that promote efficient APC–T-cell interactions. By contrast, muscle cells do not express these costimulatory molecules and so cannot function as effective APCs. In addition, APCs express cytokines that co-ordinate the immune response.

Although some viruses, such as adenovirus, appear to infect DCs directly, gene transfer using plasmid DNA appears to generate cytotoxic T lymphocyte (CTL) responses, primarily $CD8^+$ CTLs, using a cross-priming mechanism (Ref. 50). Cross-priming refers to the uptake of exogenous proteins by the APC and presentation in the context of MHC class I as well as class II. The same is likely to be true for adeno-associated viral (AAV) and retroviral vectors. It has been suggested that the use of muscle-specific promoters might avoid the generation of immune responses by avoiding expression in APCs (Ref. 51), but this would not prevent immune responses if cross-priming is involved.

As dystrophin is a cytoskeletal molecule (and hence not accessible to antibodies), the generation of humoral immune responses by $CD4^+$ T cells is not of itself an important concern compared with the generation of cytotoxic immune responses that are predominantly driven by $CD8^+$ T cells. However, the presence of specific antibodies to dystrophin (e.g. Refs 52, 53, 54) shows that the molecule is seen as foreign by the immune system and might act as an indicator for potential cytotoxic immune responses.

Evidence for immune responses to dystrophin

Immune responses after human cell/tissue transplants in DMD and BMD patients are reviewed in Table 2. Results from transplants and gene transfer of murine dystrophin into *mdx* mice are reviewed in Table 3, and similar studies of human dystrophin transferred into *mdx* mice are summarised in Table 4. The tables include only those papers that make reference to possible immune responses, specifically humoral or cell-mediated immune responses and/or reductions in dystrophin-positive fibres over time in fully immunocompetent animals. For a more complete listing of all dystrophin gene transfer experiments using viral and nonviral vectors, see Ref. 28. In addition to the studies summarised in Table 2, 3 and 4, there is a report of immunological data from adenoviral-mediated human minidystrophin gene transfer into the GRMD dog: Howell et al. (Ref. 56) observed a decline over time in dystrophin-positive fibres accompanied by production of dystrophin-specific antibodies and the presence of a $CD4^+$/$CD8^+$ T-cell infiltrate.

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Table 2. Transplantation of cells/tissues containing human dystrophin into human recipients: evidence for immune responses to dystrophin (tab002dwl)

Experiment	Donor / Recipient	Humoral response	Mononuclear infiltrate and/or cytotoxic response	Comments	Ref.
Myoblast transfer (compatible MHC class I and class II-DR)	Human full[a] / Human	Antibodies against donor myoblasts fixed complement and lysed myotubes in culture (antigens on external surface); sera also stained C57/BL10 sections but not *mdx* Antibody titre against dystrophin and other small-molecular-weight proteins (western blot)	Not studied/reported		52
Myoblast transfer (compatible MHC class I and class II-DR)	Human full / Human	2/5 patients had antibodies against donor myotubes, which fixed complement and lysed myotubes in culture (antigens on external surface); sera also stained C57/BL10 sections but not *mdx* Antibody titre against dystrophin and other small-molecular-weight proteins (western blot)	Not studied/reported	No gain of muscle function Very low percentage of dystrophin-staining fibres 6 months after transplantation	53
Myoblast transfer (all MHC class I and class II alleles compatible)	Human full / Human	Not studied/reported	MHC class I expression on surface of non-regenerated fibres in injected muscles of all patients at 8 weeks No inflammatory cell infiltrate present in injected muscles except for very few $CD8^+$ T cells	3/8 patients showed <5% dystrophin-positive fibres at 8 weeks but none remaining by 1 year	112
Cardiac transplant	Human full / Human mini	Specific antibodies against the dystrophin sequence deleted in patient and present in the heart donor	Not studied/reported	Graft failure (non-compatible?) despite cyclosporine and prednisone treatment	54
Myoblast transfer (some MHC-compatible) plus cyclosporine	Human full / Human	Not studied/reported	Cells containing donor nuclei surrounded by host T cells in 1/6 patients at 1 month	Decrease in dystrophin-positive fibres with a donor nucleus from 1–6 months	113

[a] Full refers to the coding sequence for the full-length dystrophin protein.
Abbreviation: MHC, major histocompatibility complex.

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Table 3. Cell transplantation or gene transfer of murine dystrophin into mouse recipients: evidence for immune responses to dystrophin (tab003dwl)

Experiment	Donor / Recipient	Humoral response	Mononuclear infiltrate and/or cytotoxic response	Comments	Ref.
Co-isogenic[a] muscle engraftment (C57BL/ 10ScSn)	Mouse full[b] / 8-week-old *mdx* mouse	High titre of dystrophin antibodies detected by western blot; sera reacted with human dystrophin and several BMD isoforms	No increase in mononuclear infiltrate in transplanted muscles	Expression of dystrophin 3 months after transplantation	114
Myoblast transplantation	Mouse full / 8–12-week-old *mdx* mouse	Antibodies stained myotubes in culture but only after permeabilisation (access to inner membrane antigens); western blot showed titres of dystrophin antibodies in some mice and also to smaller proteins	Higher infiltration of $CD8^+$ cells, macrophages and NK cells in transplanted muscles compared with control, but less than in a noncompatible transplant	Antibody production did not lead to rejection (stable up to 6 months) The fewer fibres expressing dystrophin correlated with lower amount of antibody in sera	115
Myoblast allo-transplantation (C57BL/10)	Mouse full / *mdx* (age not given)	No antibodies detected	Few $CD4^+$ cells and macrophages	Many dystrophin-positive fibres at 1 week	116
Myoblast transfer: syngeneic and allogeneic	Mouse / *mdx* (age not given)	Antibodies against FCS (component of myoblast media)	In allogenic transfer $CD4^+$ and $CD8^+$ staining, and granzyme B and IFN-γ messages	In syngeneic transfer, high percentage of dystrophin-expressing fibres up to 16 weeks after transplantation; in allogeneic and syngeneic male to female transfer, reduced dystrophin-expressing fibres from 4–16 weeks	117
Allo-transplantation (C57BL/10)	Mouse full / 4-week-old *mdx* mouse	Not studied/reported	$CD8^+$ cells surrounding dystrophin-positive fibres by 6 weeks CTLs against C57BL/10 and mouse full-length transgenic myoblasts	Dystrophin-positive fibres 2 weeks after transplantation but greatly decreased by 6 weeks; myoblast survival achieved by IV immunogenic peptides 2 weeks before transplantation	118
Plasmid DNA direct injection into muscle	Mouse full and mini[b] / 6–8-week-old *mdx* and *mdx*/nude mice	Not detected	Not detected	Mice appeared tolerant to murine dystrophins (longest timepoint studied was 3 months)	57

[a] Co-isogenic indicates that mice are genetically identical except for a single locus, usually the MHC locus.
[b] ‘Full’ refers to the coding sequence for the full-length dystrophin protein; ‘mini’ refers to the coding sequence for the internally deleted Becker dystrophin protein.
Abbreviations: BMD, Becker muscular dystrophy; CTL, cytotoxic T lymphocyte; FCS, foetal calf serum; IFN-γ, interferon γ; IV, intravenous (administration); MHC, major histocompatibility complex; NK, natural killer.

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Table 4. Cell transplantation or gene transfer of human dystrophin into mouse recipients: evidence for immune responses to dystrophin (tab004dwl)

Experiment	Donor / Recipient	Humoral response	Mononuclear infiltrate and/or cytotoxic response	Comments	Ref.
Direct injection with adenoviral vector	Human mini[a] / 7-day-old *mdx* mouse	Not studied/ reported	Not studied/reported	Similar percentage of dystrophin-expressing fibres at 2, 4 and 13 weeks; possible lack of immune response	82
Direct injection with retroviral vector	Human mini / 7–8-week-old *mdx* mouse	Not studied/ reported	Not studied/reported	Similar but very low percentage of dystrophin fibres at 4–5 weeks and 4–9 months; possible lack of immune response	102
Myoblast xeno-transplantation	Human full / *mdx* mouse (age not given)	Antibodies against donor myoblasts detected by flow cytometry	$CD8^+$ and $CD4^+$ T cells at 1–3 weeks clustered around human myoblasts	Decrease in dystrophin-positive fibres from 1–6 weeks; possible immune response	116
Direct injection with adenoviral vector	Human mini / 5-day-old and 5–6-week-old *mdx* mouse	Not studied/ reported	At 2 months, mononuclear cell infiltrates (e.g. $CD4^+$/ $CD8^+$ T cells and macrophages) present around dystrophin-expressing fibres; some dystrophin-positive fibres expressed MHC class I and N-CAM	Good expression of dystrophin at 10 days and marked decrease by 2 months; decrease more acute in older mice	83
Direct injection with adenoviral vector	Human mini / 4-week-old *mdx* mouse	Antibodies against adenoviral proteins detected by ELISA in non-immuno-supressed *mdx*	Fairly high levels of $CD4^+$/$CD8^+$ T cells and macrophages in non-immunosuppressed *mdx*	Similar percentage of dystrophin-expressing fibres in both groups at 10 days but clear decrease in the non-treated *mdx* at 2 months	89
plus FK506 immuno-suppression		Antibodies against dystrophin (staining of human normal muscle with sera)		When adenoviral vector re-administered 30 days after first injection there were many fewer dystrophin-positive fibres in the FK506 group, and some mice developed antibodies to dystrophin	
Plasmid DNA direct injection into regenerating muscle	Human full / 4–8-week-old *mdx* mouse	Antibodies against dystrophin detected (western blot) at day 21 after single injection and thereafter after a second dose	Weak T-cell response against human dystrophin detected in 3/6 mice by ELISpot assay; moderate mononuclear infiltrate increased only after second dose at day 21	Maximum expression of dystrophin at 7 days and almost absent by 28 days; if a second dose administered at 21 days, the number of dystrophin-positive fibres 1 week later was not increased	58

(continued on next page)

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Table 4. Cell transplantation or gene transfer of human dystrophin into mouse recipients: evidence for immune responses to dystrophin (tab004dwl) (*continued*)

Experiment	Donor / Recipient	Humoral response	Mononuclear infiltrate and/or cytotoxic response	Comments	Ref.
Plasmid DNA direct injection into muscle	Human full and mini / 6–8-week-old *mdx* and *mdx*/nude mice	Antibodies against dystrophin detected by western blot and ELISA	CD8+ T-cell clustering around human dystrophin-positive fibres (but not murine revertant fibres)	Loss of dystrophin-positve fibres over time in *mdx* but not in *mdx*/nude mice	57
Direct injection with adeno-associated virus	Human micro / 10- and 50-day-old *mdx* mice	Not studied/ reported	No CTLs directed against myofibres (data not shown)	Widespread expression of dystrophin still present at 4–6 months	97
Direct injection with adenovirus	Human mini / neonatal *mdx* mouse	Not studied/ reported	Not studied/ reported	30% dystrophin-positive fibres at 1 month	106
	Human mini / adult *mdx* (45–60 days) mouse			13% dystrophin-positive fibres at 10 days and marked reduction by 2 months	

[a] 'Full' refers to the coding sequence for the full-length dystrophin protein; 'mini' refers to the coding sequence for the internally deleted Becker dystrophin protein; 'micro' refers to the coding sequence for a substantially internally deleted dystrophin protein.
Abbreviations: CMV, cytomegalovirus; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; ELISpot enzyme linked immuno-spot assay; MHC, major histocompatibility complex; N-CAM, neural cell adhesion molecule.

As can be seen from Table 2, 3 and 4, several studies have indeed reported evidence of immune responses to dystrophin following therapeutic strategies in dystrophic mice and humans. However, the effects of such responses are unclear in the vast majority of studies. In particular, it is not clear if the immune responses to dystrophin are primary in nature (i.e. against the protein itself) or if they are the secondary consequences of an immune response against some component of the vector system. Other potentially immunogenic proteins include the viral coat proteins, possible products of residual viral genes in some vectors (see later), variant proteins in mismatched cell transplants, and serum products (usually of bovine origin) possibly contaminating cultured cells.

Two studies designed to examine specific immune responses to dystrophin have been reported recently (Refs 57, 58). These studies used plasmid DNA for dystrophin gene transfer and so avoided the complications of co-administration of other potentially foreign proteins, as is the case with viral- or cell-based methods of gene transfer. Both studies demonstrated a humoral and cellular immune response following plasmid-based transfer of human dystrophin cDNAs that was associated with a loss of dystrophin-expressing fibres in the *mdx* mouse. The studies assessed the production of dystrophin-specific antibodies using western blots, and Ferrer et al. (Ref. 57) also used an enzyme-linked immunosorbent assay (ELISA)-based method to determine the relative titres of IgG_{2a} dystrophin-specific antibodies in *mdx* and *mdx*/nude mice following gene transfer. Both studies reported a $CD8^+$ infiltrate associated with the loss of dystrophin-positive fibres in the injected leg. In addition, Braun and co-workers were able to demonstrate a weak dystrophin-specific T-cell response in lymph nodes from mice treated with a full-length human dystrophin cDNA construct (Ref. 58). They also reported an immune response to human dystrophin gene transfer in the normal C57BL/10 mice, the strain from which the dystrophin-deficient *mdx* was originally derived. Despite the production

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of relatively low numbers of dystrophin-positive fibres after plasmid gene transfer, robust immune responses were generated. The sensitivity of the immune response might have been aided by the immunostimulatory effects of the unmethylated CpG motifs present in plasmid DNA (Ref. 59). These two complementary data sets demonstrate that, despite the 90% amino acid similarity between the human and mouse proteins, there are sufficient differences in epitopes to lead to the production of cellular and humoral immune responses. Ferrer and colleagues (Ref. 57) further demonstrated that the loss of human dystrophin-positive fibres in the *mdx* mouse was not due to toxic effects of de novo expression of dystrophin in muscle as there was no deleterious response to expression of murine dystrophin and no loss of human dystrophin-positive fibres in the immunodeficient *mdx*/nude mice. The authors speculated that the lack of an immune response to murine dystrophin might be due to the continued expression of other isoforms of dystrophin in *mdx* mice (see below).

In summary, studies on immune responses to dystrophin have shown that expression of human dystrophin in both mouse and man can generate humoral immune responses and, at least in mouse, this is accompanied by a cytotoxic immune response that can destroy the treated muscle fibres. These findings raise serious concerns about the deleterious consequences that might arise following restoration of dystrophin expression in DMD patients. However, the data of Ferrer and colleagues (Ref. 57) suggest that immune responses are not inevitable and some DMD patients might be tolerant to restoration of dystrophin expression, particularly those that, like the *mdx* mouse, have point mutations and express revertant fibres.

Tolerance to revertant fibres

As previously mentioned, the *mdx* mouse and many DMD patients express rare revertant fibres that express dystrophin. This expression is thought to be due to exon skipping in the formation of the dystrophin mRNA that leads to a restoration of the reading frame and subsequent translation into a recombinant dystrophin protein. In the case of the *mdx* mouse and DMD patients with point mutations, it is possible that the vast majority of epitopes of the full-length protein will be present in at least some muscle fibres. Revertant fibres increase in number with age and do not appear to attract the attention of the immune system. This tolerance of revertant fibres might be due to low expression of MHC class I on the muscle cell surface and/or the presence of the other isoforms of dystrophin. Indeed, the presence of revertant fibres and/or other isoforms of dystrophin might be responsible for the tolerance to the transfer of mouse dystrophin observed by Ferrer et al. (Ref. 57).

An alternative explanation for the apparent tolerance of the *mdx* mouse to the presence of revertant fibres and the expression of full-length murine dystrophin after gene transfer might be that the antigen load is below threshold owing to the rare revertant events and the low efficiency of plasmid-based gene transfer in the studies cited above. However, recent studies in our laboratory show that electrotransfer of plasmid encoding mouse dystrophin after hyaluronidase pre-treatment leads to long-term expression at high transfection efficiencies (H. Gollins, J. McMahon, K.E. Wells and D.J. Wells, unpublished).

Immune responses to vectors

Adenoviral vectors

Adenoviruses can infect both dividing and quiescent cells, including those in muscle (Ref. 60). Adenoviral gene transfer is more efficient into neonatal than mature muscle for two reasons: first, there is a high level of expression of receptors involved in viral transduction, particularly the coxsackie/adenovirus receptor (CAR) in neonatal muscle; and, second, the immune system is less mature (Refs 61, 62). Adenoviral vectors stimulate immune responses to both the virus capsid and the transgene product, although the response to the transgene is probably more significant in the case of the reporter gene β-galactosidase (Refs 63, 64). Adenoviral gene transfer in adult muscle is more successful when immunosuppressive methods are used such as antibodies to CD28 plus calcineurin (Ref. 65), depletion of $CD4^+$ and $CD8^+$ T cells (Ref. 66), or thymectomy and $CD4^+$ T-cell depletion (Ref. 67).

A study by Harvey and colleagues shows that, in human patients, who might have a pre-existing neutralising antibody titre to adenovirus, there is a correlation between the highest anti-adenoviral antibody titre obtained after vector administration and the previous titre (Ref. 68). The induction of a neutralising antibody titre did not occur in all patients and was in part dependent on the site of vector administration. The variability in pre-

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existing antibody levels will complicate the administration of adenoviral vectors for gene therapy; indeed, 55% of the population have neutralising antibody titres to adenovirus Ad5 (Ref. 69). If the efficacy of gene therapy with adenoviruses requires high-dose levels or multiple treatments, then immunosuppressive reagents such as cyclosporin A are likely to be required. The long-term effect of administration of such drugs to transplant patients includes a high incidence of cancer in surviving patients; in particular, a high incidence of skin cancer in Australian transplant patients has been reported (Refs 70, 71). Drugs such as FK506 and cyclosporin A are not only toxic in many organs but might be actively involved in the generation of tumours (Refs 72, 73).

One vector system has been developed from the early adenoviral vectors that should reduce the adverse effects because all viral genes have been eliminated. These vectors are known as gutted, high-capacity adenoviruses (HC-Ads) or pseudoadenoviruses (pAVs) (Refs 74, 75, 76, 77, 78, 79). These new-generation vectors show improved longevity of expression compared with first-generation vectors and no inhibition of promoter activity in the absence of a transgene-specific immune response (Refs 80, 81). The increased capacity of these vectors, which contain no viral coding sequences, makes them suitable for gene transfer of full-length dystrophin. Until the recent developments with high-capacity adenoviruses, the majority of gene transfer studies had been performed using minidystrophin in adenoviral vectors. Early trials of adenovirus minidystrophin showed good transduction of neonatal muscle but more-recent studies have shown poor transduction in adult muscle, with loss of expression by 60 days (Refs 82, 83, 84, 85). Indeed, the probable immune responses to the human recombinant dystrophin transgene used in preclinical gene transfer studies in *mdx* mice has been neglected in comparison with efforts to eliminate the vector-dependent immune responses.

There is a very narrow window for efficient adenoviral gene transfer without toxicity, as seen in the effect of adenoviral reporter gene transfer into the diaphragm of *mdx* mice (Refs 66, 86). Dystrophin gene transfer in adenoviral vectors also causes muscle damage with inflammation and humoral responses to the vector and transgene (Refs 87, 88). The expression from first-generation vectors might also be reduced over time as a result of promoter 'shut off', as viral genomes can be detected despite the loss of transgene expression (Ref. 81). Adenoviral minidystrophin gene transfer in conjunction with different immunosuppressive reagents has led to good long-term transduction efficiencies in adult *mdx* mice and in the dog model (Refs 56, 65, 88, 89). However, the development of these vectors for dystrophin gene transfer has been severely curtailed by recent concerns about adverse reactions to adenoviral vector delivery in human clinical trials: over 650 adverse reactions have been reported in a National Institutes of Health (NIH) survey (Ref. 90) following the death after receiving gene therapy of a boy suffering from a partial ornithine transcarbamylase (OTC) deficiency (Ref. 91). Furthermore, primate studies have also shown adverse responses to the adenoviral vectors, resulting in the death of two primates (Ref. 92). The high-capacity viruses might not circumvent the acute haematological responses to systemic vector delivery as the capsid is still present in the HC-Ad vectors. Nevertheless, the HC-Ad vectors give extremely good transduction efficiencies in adult *mdx* mice and long-term expression of either human and mouse dystrophin (unpublished data cited in Ref. 93); thus the use of adenoviral vectors for gene transfer in the treatment of DMD patients remains a very real possibility.

AAV vectors

Gene transfer into skeletal muscle with AAV is highly efficient, and AAV has been successfully used for long-term expression of human factor IX in a dog model (Ref. 94). Unfortunately, AAV has a severely limited capacity for exogenous DNA (4.5 kb). This vector system is ideally suited for the transfer of smaller genes involved in muscular dystrophies. For example, clinical trials have been initiated for sarcoglycan deficiency using AAV. This follows the successful correction of pathology in a hamster with γ-sarcoglycan deficiency that provides a model of limb-girdle muscular dystrophy (Ref. 95). Vector delivery via the femoral artery gave efficient rescue of the muscle when used together with histamine (Ref. 96). The histamine induces endothelial permeabilisation, allowing the viral particles access to the muscle fibres. Recent experiments in *mdx* mice have shown beneficial effects of transducing dystrophic muscle with an AAV carrying a microdystrophin,

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so-called because it contains additional deletions and so is smaller in size than the minidystrophin (Refs 97, 98, 99). However, as AAV is a naturally occurring infection in humans, antibody titres to AAV2 have been detected in virtually all patients screened in a study by Chirmule et al., of which 32% were neutralising antibodies (Ref. 69). Although neutralising antibodies directed against the capsid proteins might decrease the efficiency of gene transfer, AAV has the advantage that cytotoxic immune responses, even to reporter genes such as β-galactosidase, do not arise readily following gene transfer (Ref. 100). This might be due to the tropism of the virus, as AAV, in contrast to adenovirus, does not transduce APCs (Ref. 101).

Retroviral vectors

Retroviral gene transfer is inefficient even in the regenerating environment of *mdx* muscle, owing to the constraining requirement for cell division to allow proviral integration, but this method could lead to lower immune responses as non-dividing APCs are not transduced (unpublished data from Transgene SA, Strasbourg, France). Minidystrophin expression following retroviral gene transfer can persist for up to nine months and transduces the satellite cell population (Refs 102, 103). Satellite cells are quiescent myoblast cells with stem cell properties that lie closely apposed to muscle fibres. When muscle fibres are damaged the satellite cells proliferate and contribute to regenerating the damaged part of the muscle.

Clinical implications/applications

The results of the study by Ferrer and colleagues (Ref. 57) suggest that, with careful patient selection, it might be possible to avoid anti-dystrophin immune responses in early clinical trials without the need to complicate the analysis with the use of immunosuppressive drugs. Patients with small mutations in the dystrophin-coding sequence and the expression of some dystrophin, as demonstrated by western blot or immunostaining, are likely to be tolerant to dystrophin and therefore allow gene transfer of the full-length recombinant gene.

However, a substantial number of DMD patients have large genomic deletions (e.g. Ref. 104), leading to the loss of a considerable number of potentially immunogenic epitopes. These patients might not be tolerant of dystrophin gene transfer. The alternative approaches would be to immunosuppress, induce tolerance, or use the utrophin gene (the autosomal homologue of dystrophin). Immunosuppression carries the risk of increased vulnerability to infection and cancer, with the former a particular concern in patients whose respiratory system might already be compromised by the muscular dystrophy. The induction of tolerance is currently the subject of intensive research effort but suitable protocols for use in combination with gene therapy are not yet clinically proven. Utrophin is expressed normally, or at higher levels, in patients with DMD, and therefore should not generate an immune response (Ref. 105). The molecule has been shown to compensate for dystrophin in transgenic and neonatal *mdx* mice (reviewed in Ref. 28). It has been suggested that the gene transfer of utrophin would avoid the potential for immune responses associated with expression of dystrophin in a previously dystrophin-deficient patient, and one study has attempted to address this issue. The adenoviral transfer of utrophin to immunocompetent mature *mdx* animals produced significant improvement in the ability of the treated soleus muscle to contract and generate force and to resist stress-induced injury, which was not achieved with a similar minidystrophin construct as a result of an immune response (Ref. 106). However, as the minidystrophin construct used was of human origin, such an immune response was not surprising. There was no clear immune response to the miniutrophin construct, which was part mouse and part human sequence. If both constructs had been of murine sequence, it is likely, given the results of Ferrer and colleagues (Ref. 57), that there would have been no significant differences between their performances. Hence, the study of Ebihara and co-workers (Ref. 106) does not help us to anticipate possible problems of immune responses in DMD patients.

Research in progress and outstanding research questions

Transgene SA (Strasbourg, France) has started a Phase I clinical trial of plasmid gene transfer in DMD/BMD (Ref. 107). Nine DMD/BMD patients over 15 years old will be enrolled in a dose escalation trial. Data will be analysed on a patient by patient basis with substantial initial characterisation of patient genotype, phenotype and immunological status. The trial aims to examine the safety of plasmid

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administration and the likelihood of generating anti-dystrophin antibodies. It should be noted that concerns have been expressed that this trial might not yield conclusive answers because transfection of muscles in these patients is likely to be very low as a result of the paucity of fibres in the heavily fibrosed muscle: no immune response might merely indicate a failure to transfect sufficient cells.

Advances are still needed in vector design and delivery for gene therapy to offer a realistic approach to the treatment of DMD. In particular, improvements in the delivery of gene vectors to multiple muscle groups via the blood supply will be critical in establishing a clinically useful treatment.

Current work in the Wells laboratory is examining immune responses to human dystrophin gene transfer into transgenic *mdx* mice expressing various recombinant human dystrophins. Experiments have begun with gene transfer of the whole human dystrophin cDNA into transgenic mice expressing various deleted versions of the human dystrophin cDNA. Such studies might raise the intriguing possibility of linked tolerance/suppression in mice expressing only part of the human molecule.

Concluding remarks

There is clear evidence that de novo expression of dystrophin can act as an antigen capable of stimulating a cytotoxic immune response, and this is a serious problem for all therapeutic strategies that aim to restore dystrophin expression in DMD patients. This is a particular concern with the use of gene and cell therapy as immune responses to the vector or cells will increase the likelihood of destruction of treated muscles. Intra-arterial administration of AAV or plasmid vectors currently offers the best system for delivery of recombinant dystrophin to dystrophic muscle while minimising the anti-vector immune responses. Although some patients might show tolerance to restoration of dystrophin expression, it is probable that most will not. Long-term use of immunosuppressive drugs is clearly not desirable in DMD and there is a pressing need to develop clinically applicable methods of inducing tolerance to dystrophin in DMD patients.

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Further reading, resources and contacts

The Leiden Muscular Dystrophy pages for research on and/or diagnosis of Duchenne (and Duchenne-like) muscular dystrophies (DMDs) provide databases of DMD mutations, descriptions of the diseases and proteins involved, and muscular dystrophy research news:

http://www.dmd.nl/

The websites for the Muscular Dystrophy Campaign (UK), the Association Française contre les Myopathies (France), and the Muscular Dystrophy Association (USA) provide information on neuromuscular conditions, ongoing research, and patient care:

http://www.muscular-dystrophy.org/

http://www.afm-france.org/cgi-bin/afm23_Prod_afm23/login_en.jsp

http://www.mdausa.org/

A recent article in *Expert Reviews in Molecular Medicine* discusses roles for nuclear proteins in muscular dystrophy:

Stephen L. Maidment and Juliet A. Ellis (2002) Muscular dystrophies, dilated cardiomyopathy, lipodystrophy and neuropathy: the nuclear connection. Exp. Rev. Mol. Med. 30 July, http://www.expertreviews.org/02004842h.htm

Features associated with this article

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Table 4. Cell transplantation or gene transfer of human dystrophin into mouse recipients: evidence for immune responses to dystrophin (tab004dwl).

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