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Dystrophin Immunity in Duchenne's Muscular Dystrophy

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SUMMARY

We report on delivery of a functional dystrophin transgene to skeletal muscle in six patients with Duchenne's muscular dystrophy. Dystrophin-specific T cells were detected after treatment, providing evidence of transgene expression even when the functional protein was not visualized in skeletal muscle. Circulating dystrophin-specific T cells were unexpectedly detected in two patients before vector treatment. Revertant dystrophin fibers, which expressed functional, truncated dystrophin from the deleted endogenous gene after spontaneous in-frame splicing, contained epitopes targeted by the autoreactive T cells. The potential for T-cell immunity to self and nonself dystrophin epitopes should be considered in designing and monitoring experimental therapies for this disease. (Funded by the Muscular Dystrophy Association and others; ClinicalTrials.gov number, NCT00428935.)

Duchenne's muscular dystrophy is an x-linked, genetically inherited disease affecting 1 in 3500 newborn boys.¹ The disease is manifested by progressive muscle weakness that is caused by mutations in the gene encoding dystrophin. Dystrophin is a large (427-kD) cytoskeletal protein.¹ The dystrophin gene has 79 exons and is too large to fit inside a recombinant adeno-associated virus (rAAV). Therefore, we used a functional miniaturized dystrophin transgene that partially restores the generation of muscle force in murine models of Duchenne's muscular dystrophy²⁻⁴ and suggests one approach to the treatment of the disease in humans. Whether cellular immunity to therapeutic dystrophin is a barrier to successful gene therapy with rAAV vectors, particularly in patients with frame-shifting deletions in the *DMD* gene, is unknown. We investigated whether dystrophin that is newly translated from a therapeutic transgene elicits T-cell immunity in patients with Duchenne's muscular dystrophy.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

METHODS

We enrolled six boys with frame-shifting deletions in the dystrophin gene in this study, which was approved by the Nationwide Children's Hospital institutional review board (Fig. 1A). Written informed consent was obtained from the parents of all the patients. Patients 9 years of age or older also provided assent in writing. The study was initiated on March 15, 2006, and completed on April 3, 2009. Asklepios Biopharmaceutical manufactured the rAAV vector used in the study. At enrollment, four patients were receiving standard glucocorticoid therapy to delay the onset of muscle weakness.^{5,6} Treatment was continued throughout the course of the study (Fig. 1A).

All patients received intravenous methylprednisolone 4 hours before vector transfer, to reduce the potential for local inflammation. To minimize pain during the procedure, the patient was given conscious sedation during injection of the vector and while muscle-biopsy specimens were obtained. One biceps muscle was treated with an rAAV vector that incorporated a modified capsid of serotype 2 and a functional mini-dystrophin transgene, representing approximately 40% of the 11-Kb coding sequence of the human dystrophin gene (for details, see the Supplementary Appendix, available with the full text of this article at NEJM.org). Patients 1, 2, and 3 received a low dose of vector (2.0×10^{10} vector genomes per kilogram of body weight), and Patients 4, 5, and 6 received a dose that was higher by a factor of five (1.0×10^{11} vector genomes per kilogram) (Fig. 1A). Adverse events are shown in Table 1. Peripheral-blood mononuclear cells (PBMCs) were tested in an interferon- γ enzyme-linked immunosorbent spot (ELISPOT) assay for reactivity to mini-dystrophin. Three pools of 40 overlapping synthetic peptides (designated MDP1, MDP2, and MDP3) that spanned the entire mini-dystrophin sequence were used to stimulate PBMCs in the ELISPOT assay (see the Supplementary Appendix for a detailed description of methods). As expected, the dystrophin peptide pools did not stimulate an interferon- γ response by PBMCs in 12 untreated persons who did not have Duchenne's muscular dystrophy (data not shown).

RESULTS

We assessed the efficiency of gene transfer by examining biopsy specimens of vector-injected biceps muscles and untreated biceps muscles on day 42 (in Patients 1, 3, 4, and 6) or day 90 (in Patients 2 and 5). In all the patients, vector DNA was detected in amounts ranging from 0.01 to 2.56 genome copies per diploid genome in the treated muscles but was not detected in the untreated contralateral biceps muscles. Myofibers expressing mini-dystrophin protein were not detected in the two biopsy specimens that were examined on day 90, but they were detected in two of the four specimens (those from Patients 3 and 6) that were examined on day 42. With the use of an antibody (MANDYS3) that detects the N-terminal of dystrophin, we found that Patient 3 had three or four dystrophin-positive fibers, and Patient 6 had one positive fiber (data not shown). These fibers were negative for dystrophin on immunohistochemical staining with an antibody that detects the C-terminal, which is removed when dystrophin complementary DNA is miniaturized to accommodate the small insertion capacity of the AAV.

Failure to establish long-term transgene expression prompted us to measure cell-mediated immune responses to mini-dystrophin in all six patients. Mini-dystrophin-specific T cells were not detected at any time in Patients 1 and 3 (who received low doses of vector), as assessed with the interferon- γ ELISPOT assay (Fig. 1B and 1D). Patient 2, who also received a low dose of vector, had robust T-cell activity against peptide pool MDP2 on the 15th day after vector treatment (Fig. 1C). The response peaked on day 30, slowly declined during the next 3 months, and was intermittently positive after day 120 (Fig. 1C). T-cell responses were substantially delayed in Patients 5 and 6 (Fig. 1F and 1G, respectively), who

received high doses of vector, and exceeded the detection threshold of 50 spot-forming cells per 1 million PBMCs only on day 60. A T-cell response to MDP3 was unexpectedly detected before vector treatment in Patient 4 and was intermittently positive throughout 2 years of follow-up (Fig. 1E). Delivery of a high dose of the gene-therapy vector to the biceps muscle of Patient 4 did not elicit the rapid increase in dystrophin-specific T-cell activity that was observed in Patient 2.

Cellular immunity was further characterized in Patient 2 and Patient 5 to better understand differences in the timing and duration of the response to mini-dystrophin. The delayed, transient T-cell response detected in Patient 5 on day 60 targeted three discrete epitopes. One exon-7 epitope was recognized by HLA-B*1801-restricted CD8+ T cells (Fig. 2A and 2B). CD4+ T cells recognized two epitopes that localized to exon 8 (Fig. 2C) and exon 6 (Fig. 2D). Because exons 3 through 17 were deleted from the dystrophin gene in this patient, we concluded that CD4+ and CD8+ T cells were primed by therapeutic mini-dystrophin that incorporated missing exons 3 through 12. Striking amino acid homology was observed between the exon-6 and exon-7 epitopes of dystrophin and corresponding sequences of β -spectrin and utrophin, cellular proteins that are also involved in muscle-force contraction and expressed at normal levels in dystrophic muscle. For instance, the exon-6 epitope presented by HLA-DQA1*0505 and HLA-DQB1*0301 (Fig. 2E) was mapped to a segment of dystrophin composed of 10 amino acids (Fig. 2F, with amino acids denoted by single-letter symbols) — 163-WSDGLALNAL-172 — that differed from β -spectrin at only two positions (Fig. 2G). T cells from Patient 5 recognized the dystrophin epitope but not the β -spectrin homologue containing the S164R and L169F substitutions, which suggests that homology between these highly related cellular proteins restricted, but did not eliminate, the pool of nonself epitopes available for immune recognition.

The rapid MDP2-specific response observed in Patient 2 was mediated by CD4+ T cells, since depletion of this subgroup from PBMCs abrogated interferon- γ production (Fig. 3A). The epitope was localized to amino acids 2809 to 2829 of exon 57 (Fig. 3B). Exon 57 should be frame-shifted because of the exon-50 deletion carried by this patient. Alternative splicing or a second mutation in the *DMD* gene can restore in-frame expression of functional dystrophin in rare revertant muscle fibers.⁷ Revertant fibers were visualized in the muscle specimen from Patient 2 with the use of antibodies directed against dystrophin exons 55–56 and 59 (Fig. 3C). Exon 57 encoding the T-cell epitope was therefore expressed in the correct reading frame, despite the exon-50 deletion. We postulated that T cells spontaneously primed by revertant fibers were present before treatment with vector and accelerated the pace of the cellular immune response to therapeutic mini-dystrophin. Indeed, a robust interferon- γ response was detected when cryopreserved PBMCs collected before and after vector treatment were stimulated with the peptide epitope spanning amino acids 2809 through 2829 of exon 57 (Fig. 3D). Dystrophin-specific antibodies were not detected in the serum of any study patients, including Patient 2 (data not shown).

The preexisting cellular immunity to dystrophin in Patient 4, who had a deletion at exons 49 through 54 (Fig. 1A and 1E), was also associated with the expression of revertant muscle fibers. The response mediated by CD8+ T cells (Fig. 1 in the Supplementary Appendix) was directed against MDP3 that contains peptides from the R24, H4, and cysteine repeat regions encoded by exons 59 through 70 (Fig. 1E). Revertant fibers expressing exons 59 through 70 were detected in biceps muscle with the use of exon-specific antibodies; in-frame dystrophin sequences containing this epitope were expressed in the skeletal muscle of Patient 4 (Fig. 1A, and Fig. 2 in the Supplementary Appendix). Expression of HLA class I proteins was greater in the treated biceps muscle than in the untreated muscle in Patient 4, indicating the potential for CD8+ T-cell recognition of myocytes (Fig. 3 in the Supplementary Appendix). A similar increase in HLA class I expression by myocytes was observed in Patient 5, in

whom CD8+ T cells were directed against nonself epitopes encoded by the transgene (Fig. 3 in the Supplementary Appendix).

DISCUSSION

The influence of cellular immunity on the outcome of human gene therapy with rAAV vectors is not yet well defined. T-cell immunity against the AAV capsid was temporally associated with the loss of transgene expression of human clotting factor IX from the liver of one patient with hemophilia B who was treated with an AAV vector.^{8,9} Capsid-specific T-cell responses are also sometimes primed by the delivery of rAAV vector to muscle^{10,11} but do not necessarily cause loss of transgene expression.¹¹ In the present study, findings in Patient 5 document the potential for target-cell destruction by T cells directed against nonself epitopes encoded by the therapeutic transgene. In other studies in humans, transgene expression has not been associated with a cellular immune response against the therapeutic protein.¹¹⁻¹⁶ Gene transfer to the eye in patients with Leber's congenital amaurosis^{12-15,17} may have been successful in part because the targeted tissue is considered to be protected by immune privilege. In other studies, the risk of T-cell immunity may have been minimized by the limited number of sequence differences between the defective self gene and the therapeutic transgene, either because the disease phenotype was caused by a small number of missense mutations or because the study patients, through careful selection, did not have large frame-shifting deletions such as those that are characteristic of Duchenne's muscular dystrophy.

Detection of spontaneously primed T cells in the blood of Patients 2 and 4 before they were treated with vector was unexpected. The rapid T-cell response that we observed after therapeutic gene transfer in Patient 2 is consistent with a memory response boosted by the mini-dystrophin protein. Why the response was not boosted in Patient 4 is unknown, but mini-dystrophin expression may have been inadequate to provoke a recall response. Muscle inflammation is an unexplained complication of Duchenne's muscular dystrophy. Glucocorticoid therapy prolongs the ability to ambulate by 1 to 2 years in patients with the disorder,^{5,6} possibly because of an antiinflammatory effect. Oligoclonal T-cell populations are a major component of the inflammatory infiltrate, which suggests that immunopathogenesis may be driven at least in part by one or more unidentified antigens.^{18,19} Whether revertant dystrophin initiates or sustains muscle inflammation remains to be determined, but the presence of spontaneously primed T cells in Patients 2 and 4 indicates that these mutant proteins do not necessarily induce tolerance as proposed.^{20,21} Why dystrophin-specific T cells were not detected in the other four study patients before vector treatment requires further study.

If dystrophin-specific T cells are highly localized to the site of inflammation, examination of muscle specimens is necessary to fully define the scope of the response in patients with Duchenne's muscular dystrophy. Conditions for antigen priming of self-reactive T cells might also vary among patients with the disease. T cells that recognize self-antigens in multiple sclerosis may be primed by cross-reactive microbial or viral antigens.²² This mechanism of T-cell priming may also be active in Duchenne's muscular dystrophy but is unlikely because at least two different environmental antigens would be necessary to elicit cellular immunity against the distinct dystrophin epitopes spontaneously targeted by Patients 2 and 4. If dystrophin is antigenic, as this study suggests, T-cell priming might also depend on the timing of spontaneous gene splicing or mutation events that generate revertant fibers⁷; furthermore, it might depend on how the timing of the events is coordinated with maturation of the immune system and tolerance induction. Finally, variation in the priming or strength of spontaneous T-cell immunity against dystrophin may be influenced by

immunogenetics (e.g., HLA class I or II) or the severity of muscle inflammation among patients with Duchenne's muscular dystrophy.

Whether circulating dystrophin-specific T cells infiltrated muscle in the study patients is unknown. Vector-injected and untreated biceps muscles contained inflammatory CD4+ and CD8+ T cells that were typical of Duchenne's muscular dystrophy. Failure to detect mini-dystrophin in biopsy specimens of the injected biceps, except in the specimens from two patients that were examined early (on day 42), is consistent with transient transgene expression due to immune elimination by T cells targeting self and nonself dystrophin epitopes. Circulating antigen-specific CD8+ T cells from Patients 4 and 5 clearly had the potential to engage myocytes, because expression of HLA class I antigens was enhanced in skeletal muscle. Myocytes visualized in the biceps muscle of Patient 2 did not express HLA class II proteins (data not shown); therefore, direct recognition of these cells by dystrophin-specific CD4+ T lymphocytes seems unlikely. Nevertheless, dystrophin-specific production of interferon- γ suggests that these CD4+ T cells are type 1 helper T cells that could potentiate muscle inflammation. Since dystrophin-specific antibodies were not detected in the serum of any study patients, including Patient 2, a role for humoral immunity against the transgene product in the outcome of gene therapy is unlikely.

Exon skipping²³ and suppression of stop codons²⁴ are also being considered as ways of increasing dystrophin expression in patients with Duchenne's muscular dystrophy. These strategies might also be expected to prime or recall dystrophin-specific T-cell responses. Indeed, we recently detected dystrophin-specific T cells in the blood and skeletal muscle of one patient immediately after treatment with gentamicin to enhance read-through of nonsense mutations in the dystrophin gene (unpublished data). The unexpected recall of autoreactive dystrophin-specific T cells suggests that the monitoring of cellular immune responses should be a priority for any experimental therapy designed to increase the number of dystrophin-positive myofibers in patients with Duchenne's muscular dystrophy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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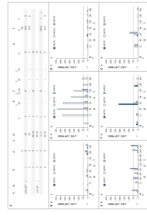


Figure 1. Dystrophin-Specific Immune Responses in the Six Study Patients

Panel A shows the vector dose (in vector genomes [vg] per kilogram of body weight) received by the patients, their age, exon deletions, and glucocorticoid regimens. Deflazacort, used by Patient 1, was not prescribed, because it is not approved by the Food and Drug Administration for use in the United States. It was obtained by the patient's family directly from Masters Pharmaceuticals in the United Kingdom. Dystrophin exons detected in revertant fibers with exon-specific antibodies are noted with a plus sign. T-cell responses are shown for Patients 1 through 6 in Panels B through G, respectively. Peripheral-blood mononuclear cells (PBMCs) collected at the indicated times were cultured with three pools of synthetic peptides (MDP1, MDP2, and MDP3) spanning mini-dystrophin domains (see the Supplementary Appendix); interferon- γ production was assessed 36 hours later with the use of an interferon- γ enzyme-linked immunosorbent spot (ELISPOT) assay. Data are presented as interferon- γ spot-forming cells (SFCs) per 1 million PBMCs. The dashed lines represent the threshold for a positive assay response (50 SFCs per 1 million PBMCs).

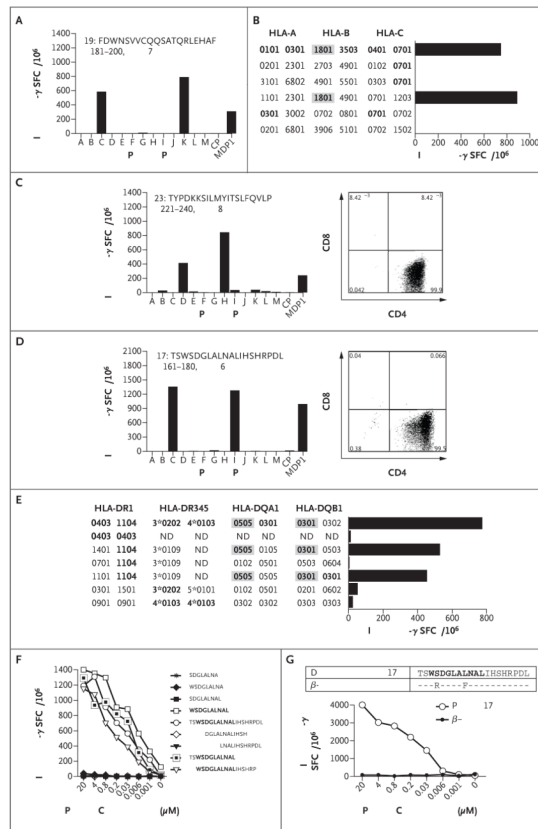


Figure 2. Characterization of Dystrophin-Specific Cellular Immune Responses in Patient 5 CD8⁺ T cells that were specific to the MDP1 pool of synthetic peptides and were detected after vector treatment, on day 60, were tested for recognition of intersecting mapping subpools (see the Supplementary Appendix). Only MDP1 subpools C and K stimulated interferon- γ production by the CD8⁺ T cells, localizing the epitope to shared peptide p19 that spanned amino acids (aa) 181 through 200, which were encoded by dystrophin exon 7 (Panel A). CD8⁺ T cells recognized only semimatched target cells that shared HLA-B*1801 expression with Patient 5 (Panel B). Two other dystrophin-specific T-cell populations expressed the CD4 protein, as assessed by flow cytometry (Panels C and D), and targeted peptide p23 contained in subpools D and H (amino acids 221 through 240, exon 8) (Panel C) or peptide p17 contained in subpools C and I (amino acids 161 through 180, exon 6) (Panel D). The p17 epitope was presented by the HLA-DQA1*0505 and DQB1*0301 class II molecules, as assessed by interferon- γ ELISPOT assay with the use of a panel of HLA-semimatched antigen-presenting cells (Panel E). The p17 epitope was further mapped to amino acids 161 through 180 of exon 6 (WSDGLALNAL) with the use of varying concentrations of the indicated synthetic peptides (Panel F). CD4⁺ T cells were stimulated with varying concentrations of dystrophin peptide p17 or the indicated β -spectrin peptide in an interferon- γ ELISPOT assay (Panel G). CP denotes control peptide pool, PBMCs peripheral-blood mononuclear cells, and SFCs spot-forming cells.

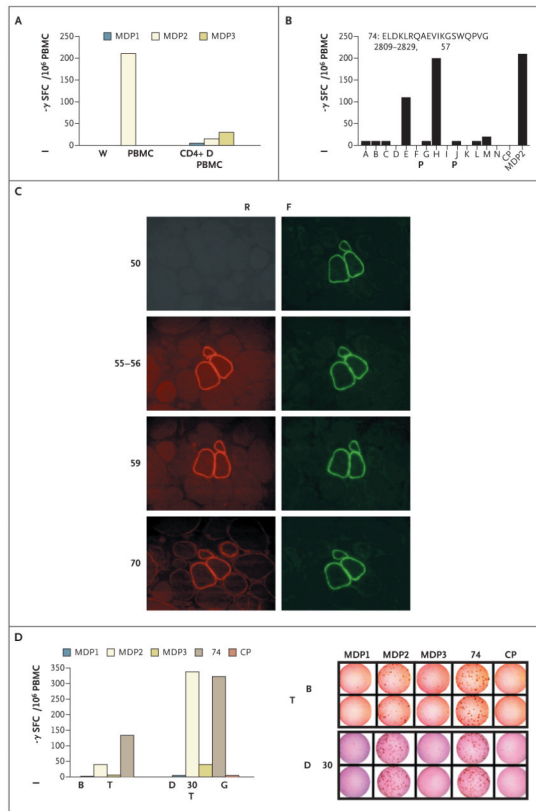


Figure 3. Mini-Dystrophin Cellular Immune Responses in Patient 2

Whole peripheral-blood mononuclear cells (PBMCs) or CD4+ T-cell-depleted PBMCs were assessed on day 60 for interferon- γ spot-forming cells (SFCs) with the indicated dystrophin peptide pools (Panel A). Intersecting MDP2 subpools E and H that stimulated an interferon- γ response contained a single common peptide, peptide p74 (amino acids [aa] 2809 through 2829, exon 57, rod domain 23) (Panel B). Revertant fibers in the muscle-biopsy specimen obtained on day 90 were detected by means of staining with the MANDYS2 antibody, which recognizes the C-terminal of dystrophin (Panel C, images on the right), or with antibodies specific for epitopes encoded by the indicated dystrophin exons (Panel C, images on the left). Interferon- γ T-cell responses were compared 1 week before treatment and on day 30 after gene transfer with the use of the three pools of synthetic peptides (MDP1, MDP2, and MDP3), peptide p74, and the control peptide pool (CP), consisting of enhanced green fluorescent protein (Panel D, left). Images of interferon- γ -producing SFCs before and after vector treatment are shown (Panel D, right). Each dot represents an interferon- γ SFC after stimulation of duplicate PBMC cultures with the indicated dystrophin peptide pools, peptide p74, or control peptides. The experiment was repeated three times with the same outcome. Antibodies used in this experiment were provided by Professor G.E. Morris.

Table 1

Mild Adverse Events in Six Patients.*

Adverse Event	No. of Patients	No. of Events
Nausea or upset stomach	1	1
Macular rash	2	2
Fungal rash	1	1
Sore throat	2	2
Total [†]	5	6

* No moderate or severe adverse events were reported.

[†] One patient had both a macular rash and a fungal rash.