INHERITED DISEASE

RESEARCH ARTICLE

Immune rejection of human dystrophin following intramuscular injections of naked DNA in mdx mice

S Braun, C Thioudellet, P Rodriguez, D Ali-Hadji, F Perraud, N Accart, J-M Balloul, C Halluward, B Acres, B Cavallini and A Pavirani

Transgène S.A., 11 rue de Molsheim, 67082 Strasbourg Cedex, France

Intramuscular administration of plasmid expressing full-length human dystrophin in dystrophin-deficient adult mdx mice resulted in humoral and weak specific T cell responses against the human dystrophin protein. Following plasmid injection, human dystrophin was detected in the injected muscles at 7 days, but decreased thereafter. Anti-dystrophin antibodies were found 21 days following plasmid injection, which coincided with transient myositis. This immune rejection

prevented the mice from expressing human dystrophin after a second plasmid injection. No anti-DNA antibodies were found. Anti-dystrophin antibodies were seen in a smaller proportion of plasmid-injected dystrophin-competent C57BL/10 mice, suggesting that the immune rejection of dystrophin may be explained partially by species differences in the dystrophin protein. Gene Therapy (2000) 7, 1447–1457.

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Introduction

Duchenne's muscular dystrophy (DMD) is a common X-linked disorder caused by a defect of the protein dystrophin.¹ Among requirements for successful gene therapy for DMD and other types of muscular dystrophy, long-term therapeutic gene expression must be achieved. Studies to determine the type, amount, and distribution of dystrophin needed for therapy have been approached by generating transgenic animals that express various forms of dystrophin on an otherwise dystrophin-deficient background, and by physical gene transfer using various viral and nonviral vectors (for a review see Refs 2 and 3). A genetic model for human DMD and for gene therapy of DMD is the dystrophin deficient *mdx* mouse which contains a nonsense point mutation in the dystrophin gene.^{4,5}

DMD gene therapy using either the full-length dystrophin cDNA or the Becker muscular dystrophy minidystrophin cDNA⁶ is still in the experimental stage in animal models. In addition to safety concerns raised by the vectors and administration protocols, the potential risks of gene therapy must be considered with respect to the transgene's own immunogenicity. Dystrophin could be considered as a neoantigen in the dystrophin-deficient animals and DMD patients. The various animal studies have shown that immunosuppression is required to achieve prolonged transgene expression in adult or young adult immunocompetent animals (see for example, Lochmüller *et al*⁷). The humoral and/or cellular response against transgenes and viral or cellular surface antigens has been studied using mainly viral or cellular

(engraftments of healthy or transduced myoblasts) vectors, which may well interfere with the immunogenicity of the transgene itself including dystrophin.^{8–10} In the case of DMD, the only clinical trials performed to date involved healthy myoblast graft.^{11–18} In these trials, which involved a total of 60 patients, the heterologous cells were obtained from healthy donors, either HLA compatible or incompatible;^{16,18} one trial involved homozygous siblings.¹⁹ An additional 150 patients were involved in further studies performed,²⁰ which however were controversial.²¹

Some of those clinical studies reported immune response as shown by detection of antibodies (Ab) against the engrafted cells,^{13,18,19} even in the case of HLA-compatible patients,¹⁶ and against dystrophin.^{16,18} Some, but not all, of the patients tested displayed anti-dystrophin Ab as shown by Western blot techniques, which however were of low sensitivity. In another study,¹³ T lymphocyte infiltration was found in one out of the six patients enrolled. Similar features were reported in the *mdx* mouse model after allo- or xenogenic cell graft^{10,22,23} or after viral vector administration.^{24,25}

Myoblast graft presents an unfavorable context from the immunological point of view, since in addition to the transgene itself, the cell vector as well as multiple culture medium protein factors, which bear many antigenic determinants, may trigger immune rejection. Despite this immune barrier, improvement of muscle strength was reported admittedly in few of the myoblast graft clinical trials.^{13,18}

The immune factor does not seem to be detrimental to the cell graft approach. There is no correlation between the immune responses observed and the number of dystrophin-expressing muscle fibers or improvement of muscle strength in the treated patients.¹⁶ In the study by Gussoni *et al*¹³ the immune response found in the only

positive patient (out of the six treated patients) did not prevent long-term expression of normal dystrophin nor survival of engrafted cells 6 months after transplantation, this with no immunosuppressive treatment. In another study a very long-term expression (6 years) was observed.²⁰ Conversely, immunosuppressive co-treatment used in some of the clinical studies did not improve notably myoblast graft efficiency.^{12,15,17}

Unlike viral vectors, naked DNA offers the advantage of being devoid of potentially immunogenic peptides or proteins. Skeletal and cardiac muscles have the ability to take up and express naked plasmid DNA injected into the extracellular space *in vivo*,²⁶ and experiments performed in *mdx* mice have shown uptake and expression of the full-length dystrophin cDNA.^{8,27} Prolonged expression of non- or poorly immunogenic foreign transgene was demonstrated using intramuscular (i.m.) administration of naked DNA, with the example of a 19-month expression of a luciferase reporter gene construct introduced directly into mouse muscles.²⁸ This has been thought to be due to the fact that mature muscle fibers are postmitotic, and thus expression of the episomally located plasmid DNA can continue for prolonged periods of time. On the other hand, DNA-based immunization of mice by i.m. injection of antigen-encoding plasmid DNA was shown to result in immune responses which may be sustained for extended periods of time without an antigen boost.²⁹

In this study, we investigated the potential anti-dystrophin reactions in *mdx* and C57BL/10 healthy mice after i.m. administration of adenoviral, as well as plasmid vectors carrying respectively the mini- or full-length human dystrophin cDNAs.

Results

Source of dystrophin antigen

We have developed a Western blot anti-dystrophin Ab detection assay derived from previous protocols from Bittner *et al*³⁰ and Vilquin *et al*.³¹ The Western blot method permits the verification that the dystrophin protein antigen being recognized by the putative Ab has the correct molecular weight (427 kDa). ELISA assays aimed at estimating the relative amount of dystrophin have been developed using healthy muscle extracts.^{32–34} Such assays could be adapted to anti-dystrophin Ab detection. However, as the purified dystrophin was not available, the use of muscle extracts as the source of antigen could lead to high background, especially with dystrophin representing only 0.002% of total proteins.¹

Each blood sample was analyzed using dystrophin-positive and dystrophin-negative Western blot strips. Sera were tested first on extracts prepared from normal and DMD human skeletal muscles as the biological source of antigen as described earlier.^{13,30} The sera used in the set-up experiment (Figure 1) were from a mouse injected i.v. with 5×10^8 p.f.u. pRSV-adenovirus human minidystrophin. We noted that background staining was not similar between the two muscle extracts which renders identification of very weak signals difficult (Figure 1). To minimize the Western blot background and variability, we used a cell culture source of antigen. In order to lower the risk of non-specific binding, anti-dystrophin Ab were investigated using antigens of a

Figure 1 GRMD cell muscle extracts allow lower background for anti-dystrophin Ab detection. Lanes 1, 6, 10: healthy human muscle extract (HHM); lane 9: DMD muscle extract; lanes 2–5 and 7, 8: GRMD cell extracts. (–): cells transfected with backbone plasmid pTG11022; (+) cells transfected with pCMV-human dystrophin plasmid pTG11025. Amount of protein extract used was 30 μ g in lanes 1–3, 6–10, and 15 μ g in lanes 4 and 5. Dystrophin was detected using monoclonal Ab NCL-DYS1 (lanes 1–5) at dilution 1/200, or *mdx* mouse serum collected 3 days (lanes 9, 10) or 21 days (lanes 6–8) after adenovirus minidystrophin (Ad-minidys) i.v. administration. Note that sensitivity seems better with cell extracts (stronger band at 427 kDa (arrows)).

different species (dog cells to assess mouse sera). Primary cultures of dystrophin-deficient GRMD dog myoblasts were prepared from a diaphragm biopsy (which were found to give rise to enriched myoblast cultures and easily transfectable cells)³⁵ of a 1-day-old male GRMD dog. The vast majority of the mononucleated cells obtained were myoblasts as assessed by anti-desmin immunocytological staining (results not shown). Batches of 100-mm Petri dish cultures were transfected with either the pCMV-human dystrophin plasmid (pTG11025) or the backbone plasmid (pTG11022) as negative control. Using a monoclonal anti-dystrophin Ab (NCL-DYS1) a dystrophin band was obtained from pTG11025-transfected cells, which was stronger than when using crude muscle extracts. This allowed us to load our gels with lower amounts of proteins (15 μ g instead of 30 μ g) to reduce the background further (Figure 1). The same result was found with the serum from an *mdx* mouse injected i.v. with minidystrophin adenovirus.

As a control experiment, we analyzed blood samples collected from five *mdx* mice at various time-points following i.v. injection of 5×10^8 p.f.u. of minidystrophin adenovirus. Anti-dystrophin Ab were seen as early as day +3 (Table 1).

Immune rejection of human dystrophin following plasmid administration

Experimental design: Vectors were injected into both the right and left TA muscles of adult dystrophin-deficient mice. Three days before vector administration, the

Table 1 Detection of anti-dystrophin Ab in mdx and C57BL/10 mice

Mouse strain	Vector/Injection route	Number of mice with anti-dystrophin Ab/total number of mice tested						
		PI	0	3	7	21	28	42
mdx	Ad-minidys/i.v.			2/5		3/3		
	pTG11025/i.m.	0/18			0/5	5/5	5/5	4/4
mdx	Ad-minidys/i.m.	0/2			0/2	2/2	2/2	2/2
	PBS/i.m.	0/14			0/5	0/5	0/5	0/4
	naive	0/3	0/3					
C57BL/10	pTG11025/i.m.	0/4			0/4	1/3	1/3	
	PBS	0/2			0/2	0/1	0/1	
C57BL/10	pTG11025/i.m.	0/3			0/3	0/3	0/3	0/3
	NaCl	0/1			0/1	0/1	0/1	0/1

PI, preimmune sera; 0–42, sera collected at various time-points (days) after vector or buffer (PBS or NaCl) administration; Ad-minidys, human minidystrophin adenovirus.

All tested animals are listed (one C57BL/10 mouse in each of the two experiments were not tested for technical reasons).

injected muscles were pretreated with notexin which induces necrosis followed by muscle regeneration.³⁶

Mdx mice: Two groups of animals were compared: (a) mice receiving 17.5 µg/50 µl per muscle of pCMV-full-length human dystrophin plasmid pTG11025 and (b) mice injected with 50 µl per muscle of the formulation buffer PBS. Each group was divided into subgroups of five mice corresponding to the different time-points, day 0 (no injection), day +7 (7 days after plasmid injection), day +21, day +28 and day +42. Four of the subgroups received a second injection on day +21 (same dose and same muscles) of either dystrophin plasmid or PBS buffer and were analyzed at day +28 or day +42. As a control two additional mice were injected into both the right and left TA muscles with 8.5×10^7 p.f.u./50 µl per muscle of the E1/E3 deleted pRSV-human minidystrophin adenovirus. At day +21, the two mice received a second injection in each TA of 8.5×10^6 p.f.u. of the viral vector.

For the normal C57BL/10 mice, in a first experiment, two groups of five mice received a single injection of either 17.5 µg/50 µl pTG11025 per TA muscle or PBS (50 µl). From each group, three animals received a second identical injection at day +21. In a second experiment, four C57BL/10 mice were injected at day 0 and day +21 with 25 µg/25 µl/TA muscle of dystrophin plasmid prepared in 0.9% NaCl solution, whereas a control mouse was treated with the 0.9% NaCl solution (25 µl per injection per TA muscle).

The animals were killed at various time-points, day 0, day +7, day +21, day +28 and day +42, and the sera and organs were collected (see Materials and methods). Muscles were assessed for dystrophin immunohistochemistry (mdx and C57BL/10 mice) and hematoxylin-eosin staining (mdx mice).

Ab against human dystrophin: Each individual serum was analyzed at a 1/50 dilution and compared with its corresponding preimmune serum. Sera from naive C57BL/10 mice were used as negative control. Representative data are shown in Figure 2. Anti-dystrophin Ab were found neither in naive C57BL/10 mouse sera, nor in the PBS or NaCl-injected mdx and C57BL/10 mice. Strong anti-human dystrophin bands were obtained with sera of all the assayed plasmid-injected mdx mice from day +21 and thereafter (see also Table 1). The Western blot bands were

seen at around the 427 kDa molecular weight (also stained with monoclonal an NCL-DYS1), which shows that the antigen labeled was indeed dystrophin. A second band at around 140 kDa was sometimes observed (see Figure 2), which corresponds probably to antigen degradation products, as it was also stained by the anti-C-terminus monoclonal Ab NCL-DYS2.

The intensity of the positive signals seemed similar between the various time-points following plasmid injection. However strict comparisons cannot be performed as it is not possible to analyze all the sera simultaneously using the Western blot technique.

A humoral response was also seen at day +21 in the minidystrophin-adenovirus i.m.-treated mice (Figure 2).

For dystrophin immunohistochemistry, NCL-DYS3 monoclonal Ab was used in parallel with MANDRA1 (Figures 3 and 4) in order to discriminate between the endogenous mouse and heterologous human dystrophins.³⁷ In mdx mice, rare dystrophin positive (called 'revertant') fibers can be found in the dystrophic muscles, consecutively to RNA splice events.³⁸ NCL-DYS3 Ab does not stain mouse dystrophin.³⁷ Therefore, putative revertant fibers in mdx mice which can be seen using MANDRA1, are not detected with NCL-DYS3. As shown in Figure 4, in PBS-injected samples, around 20 MANDRA1 positive fibers were detected, which most probably correspond to revertant fibers, as very few if no positive fibers were found using NCL-DYS3. Immunohistological analysis indicated that dystrophin expression was maximal at 7 days with a mean of 60 human dystrophin positive-fibers per muscle section ($n = 10$ muscles) as seen with NCL-DYS3 mAb (Figure 4). It is not excluded that mock injections may give rise to new revertant fibers through a necrosis/regeneration process, which may explain the differences in the number of revertant fibers at day 0 (virtually no revertant fibers) and in the time-points following plasmid injections. At day +28, the number of human dystrophin-expressing fibers decreased despite re-administration of the dystrophin plasmid. This indicates that re-administration failed to allow re-expression of the transgene.

Only one C57BL/10 mouse (injected twice with 17.5 µg plasmid into the TA muscles) tested displayed anti-dystrophin Ab, and from day +21 on (Figure 5 and Table 1). However, the average number of human dystrophin-expressing muscle fibers in the plasmid-injected animals

Figure 2 Humoral immune response in *mdx* mice following i.m. administration of pCMV-human full-length dystrophin plasmid or adenovirus-human minidystrophin. Ab were detected using 15 μ g protein extracts from GRMD cells transfected with either backbone plasmid pTG11022 (–) or pCMV-human full-length dystrophin plasmid pTG11025 (+). Each Western blot shows data from sera of two representative *mdx* mice and one naive C57BL/10 mouse at various time-points (0 to 42 days after injection). NCL-DYS1, staining of human dystrophin expressing GRMD cell extract with NCL-DYS1 monoclonal Ab diluted at 1/200 (positive control), PI, preimmune sera; I, immune sera. Bottom right, Western blot shows the kinetics (over 42 days after vector injection) of anti-dystrophin Ab detection of one *mdx* mouse injected i.m. with adenovirus minidystrophin (Ad-minidys). Sera were analyzed at 1/50 dilution. Arrows indicate the band corresponding to 427 kDa dystrophin. *A 140 kDa product.

was lower than for *mdx* mice (with NCL-DYS3) (mean of 10 fibers per section, $n = 4$ muscles). Differences in transfer efficiencies may account for the contrasting Ab responses seen between the normal and the dystrophin-deficient strains. Injections of a higher amount of plasmid ($2 \times 25 \mu\text{g}$) were not found to be more immunogenic. Another explanation may be in the ability of the C57BL/10 mice to express dystrophin constitutively which may prevent immune rejection of exogenous dystrophin.

Cellular response: Spleen cells from *mdx* mice injected 28 days previously with the human dystrophin plasmid or PBS (or NaCl) alone were assessed by ELISpot for a T

cell response to human dystrophin. The data shown in Figure 6a indicate that the response of all four mice to the negative control antigen (MVA-N33.1) are consistent. The response to the human dystrophin antigen by the PBS-injected mice was no greater than that to the control antigen. In contrast the T cell response of both pTG11025-injected mice to the MVA-human-dystrophin-infected APC is greater than that to the control MVA-N33.1 infected APCs. This indicates that a T cell response to human dystrophin can be detected in the spleen cells from pTG11025-injected *mdx* mice using this assay.

Spleen cells taken from *mdx* mice 42 days after injection, as well as lymph node cells from *mdx* mice taken 28 and 42 days after plasmid injection were also assessed (Figures 6b and c). In the experiment shown in Figure 6b, responses to the control antigen are variable making interpretation difficult, however, spleen cells from mouse No. 23 (pTG11025-injected, day +42) showed double the number of spots in the presence of the MVA-human dystrophin-infected APCs than to the PBS-infected APCs, suggesting that this mouse is also making a T cell response to human dystrophin.

In the experiment shown in Figure 6c, cells from the lymph nodes draining the site of injection were assessed for human-dystrophin-specific T cell activity by ELISpot. Two *mdx* mice injected 28 days before, and two mice injected 42 days before death were assessed. Mouse No. 19, (pTG11025-injected, day +28) whose spleen cells showed an ELISpot response in the experiment shown in Figure 6a, showed a weak but detectable T cell response in this assay. Lymph node cells from other mice showed no response at all.

A preliminary test was also carried out in which spleen cells from *mdx* mice killed 21 days after vector injection were assessed, using several experimental conditions. No difference between responses to MVA-N33.1-infected APC and to MVA-human-dystrophin-infected APC were observed (data not shown). In addition, C57BL/10 mice were injected with pTG11025 and assessed for ELISpot activity to human dystrophin. No activity was seen (data not shown).

We have thus been able to detect T cell responses to human dystrophin in three (mice Nos 18, 19 and 23) of the six *mdx* mice tested injected with pCMV-human dystrophin plasmid. The responses, however, were very weak. A response to a viral antigen such as mice injected with an MVA-HPV-E7 construct, for example, will produce several hundred spots per million spleen cells (not shown).

Cellular infiltrates into injected muscles: Expression of human dystrophin dropped after day +7 which paralleled the initiation of the anti-dystrophin Ab response. Standard histological (hematoxylin/eosin) analysis of the injected muscles was performed looking mainly for cellular infiltrates graded 0 (normal), 1 (moderate, focal infiltrates), 2 (presence of lymphocytes and histiocytes, onset of necrosis), 3 (intense myositis with degenerescence and cell necrosis). Table 2 shows moderate inflammation consecutively to plasmid injection, which returned to normal after 4 weeks. The initial (back to normal from day +7 on) strong inflammatory process is due mainly to the notexin pretreatment. A substantial myositis was found upon the second plasmid adminis-

Figure 3 Kinetics of dystrophin expression in TA muscles parallels the anti-dystrophin humoral response in *mdx* mice injected with pCMV-human full-length dystrophin plasmid pTG11025. Immunohistochemistry of dystrophin detected with MANDRA1 monoclonal Ab. Microscopic magnification 200 $\times$. pTG11025, muscles injected with pTG11025; PBS, muscles injected with PBS buffer. (–), no anti-dystrophin Ab detected by Western blot; (+), anti-dystrophin Ab detected. Arrows indicate the days of pTG11025 or PBS i.m. injections.

Figure 4 Kinetics of dystrophin expression in TA muscles of *mdx* mice injected with pCMV-human full-length dystrophin plasmid pTG11025. Data are mean $\pm$ s.e.m. of maximal number of dystrophin-expressing muscle fibers per muscle ($n=10$ values; right and left TA muscles of five mice) stained with either MANDRA1 (left panel) or NCL-DYS3 (right panel) monoclonal Ab. Empty bars, after injection of PBS buffer alone; full bars, after injection of pTG11025. Injections were performed at day 0 and day +21.

tration, and it returned to nearly normal 3 weeks thereafter.

Similar observations were made in C57BL/10 mice (not shown).

Anti-DNA Ab

A group of 10 CD1 $\times$ CD1 $\times$ (ICR)BR male mice (a strain classically used for toxicology studies) received two i.m. injections at 3-week intervals (day 0 and day +21) of the dystrophin plasmid (same injection protocol

Figure 5 Anti-dystrophin Ab found in one C57BL/10 mouse following i.m. administration of pCMV-human dystrophin plasmid pTG11025. Western blots performed with protein extracts of GRMD cells transfected with either pTG11025 (+) or backbone plasmid pTG11022 (–). C57BL/10, staining of GRMD cells with serum from a naive C57BL/10 mouse (negative control); NCL-DYS1, positive control staining with monoclonal anti-dystrophin NCL-DYS1 Ab (diluted 1/200); PI, preimmune serum of the plasmid-injected mouse. Immune sera were collected at either 7, 21 or 28 days after pTG11025 first injection (a second injection was performed at day +21). Sera were used at dilution 1/50. For this particular assay, higher background was seen due to the recourse to 30 μ g of protein extracts which displayed lower amounts of human dystrophin following transfection with pTG11025 as compared with usual batch.

as described above). Sera were collected at day 0, 28 and 36.

A group of control animals was injected with PBS buffer and maintained and sampled in the same conditions (study number 16986 TSS, CIT, Evreux, France). No toxicity signs were observed. The day +28 and day

Figure 6 Weak human dystrophin-specific T cell response found in *mdx* mice. Mice were injected either with PBS buffer (PBS) or pCMV-human dystrophin plasmid pTG11025. Empty bars, negative control EL4 APC cells infected with MVA-N33 (no dystrophin insert); full bars, assay performed with EL4 cells infected with MVATG-14212 (carrying human full-length dystrophin cDNA). Bars are mean $\pm$ s.e.m. of four determinations (four wells per individual measurement). Individual (numbered) *mdx* mice were injected either with PBS alone (PBS) or with pTG11025. Spleens (a, b) and lymph nodes (c) were collected upon death of the animal at either 28 (day +28) or 42 (day +42) days after vector or buffer administration (i.m. injections at day 0 and day +21).

Table 2 Cellular infiltration is found transiently in the TA muscles of pCMV-human dystrophin plasmid pTG11025 injected in *mdx* mice

Condition	Dystrophin expression	Myositis grade
Day 0 (PBS)	No	2
Day +7 (PBS)	No	0
Day +7 (pTG11025)	Yes	1
Day +21 (PBS)	No	0
Day +21 (pTG11025)	Rare positive fibers	1
Day +28 (PBS)	No	1
Day +28 (pTG11025)	Very sparsely dystrophin positive fibers	2/3
Day +42 (PBS)	No	0
Day +42 (pTG11025)	No	1

Hematoxylin/eosin staining performed at various time-points (day 0 to day +42) following injections (done at day 0 and day +21) of either PBS or pTG11025.

+36 sera samples of pTG11025 injected and PBS control animals were assayed for the presence of anti-DNA Ab.

No tested sample appeared positive even at the lowest level (sensitivity level $<3 \mu\text{g/ml}$). Spiking of the positive control with tested mice sera did not inhibit the reaction, in fact, a slight stimulatory effect was detected.

It thus appeared that in CD1®Crl:CD-1®(ICR)BR mice, pTG11025 doses known to allow dystrophin expression in *mdx* mice did not promote the appearance of anti-DNA Ab, even after repeated reinjection.

Discussion

We have observed that i.m. administration in *mdx* mice of pCMV-human dystrophin plasmid elicited humoral and cellular immune responses against the newly produced human protein. This seemed to contribute to rejection of the muscle fibers which expressed the human dystrophin protein and prevented successful readministration. No immune response was seen in non-injected or buffer injected animals. Only one of seven plasmid-injected C57BL/10 normal mice developed detectable immune response.

This is to our knowledge the first report of a specific study of immune response towards human dystrophin following administration of naked DNA carrying the full-length human dystrophin in *mdx* mice (see also Ferrer *et al* (this issue, pp 1439–1446). Studies in C57BL/6 and BALB/c mice injected with plasmids reported ELISA-measured Ab directed against the transgene (human factor VII or HBV envelop protein) starting 7 to 10 days following injection.^{29,39} In the later study, the authors relate the humoral response to the nature of the transgene and suggested that it might be potentiated in a regenerating muscle.²⁹

Intramuscular injection of plasmid DNA in mice was found sometimes to trigger anti-DNA Ab. However, this humoral response is unable to elicit any glomerulonephritic or autoimmune-like effect, even in mice which are very sensitive to lupus.⁴⁰

Also, induction of anti-DNA Ab is found preferably when the plasmid vector is complexed with proteins.⁴¹ In human vaccine clinical trials, no anti-DNA nor elevation of muscle enzymes was found in the patients. In the present study we found no anti-DNA abs following plasmid administration.

Intramuscular administration of plasmid vectors was not reported to be associated with autoimmune features even in very susceptible animals.⁴⁰ To date, no induction of autoimmunity has been reported using plasmids in humans. Plasmid DNA is being considered as the simplest and safest vector currently available.²

As reported by us and others, immune responses triggered by gene transfer depend on both the transgene and the nature of the vector.^{42,43} In the case of intramuscular administration of plasmid DNA, the fate of transgene expression seems often to be linked to an immune response directed towards the transgene itself rather towards the vector,⁴⁴ as suggested by data on persistence of the vector DNA or mRNAs in the injected muscles.^{24,45}

Various studies reported an anti-dystrophin immune response following intramuscular injections of vectors, especially viruses.^{8,27,42} This could be explained at least in part by the expression in mice of a human dystrophin protein. This foreign antigen-specific response is supported by our present study and further substantiated by our observation that there was no decrease of the amount of revertant fibers following human dystrophin plasmid administration. It is also supported by the report by Ferrer *et al* (pp 1439–1446) using intramuscular injections of a plasmid carrying the human minidystrophin gene which induced anti-dystrophin Ab and lymphocyte infiltration in *mdx* mice, whereas no immune response was found with plasmids carrying either the mouse full-length dystrophin or minidystrophin gene.

It has been suggested that revertant fibres might induce immune tolerance for the neoantigen. The amount of revertant fibers seems to increase with age in humans,⁴⁶ as well as in the *mdx* mouse model,⁴⁷ which leads to the supposition that the potential anti-dystrophin response does not induce massive destruction of the muscle fibers.

However, previous results indicated that Ab were raised against dystrophin after myoblast or muscle tissue allotransplantation into *mdx* mice.^{30,31} This was also described for humans.^{13,48} Production of a low level of Ab cannot be excluded if revertants appear late in development. This response could be further boosted by injecting a dystrophin vector. No Ab were found in pre-immune sera in the present study. However, we cannot exclude that Ab against mouse dystrophin might have remained undetected as we used human dystrophin as antigen.

Anti-human dystrophin immune response was detected in spite of a close homology between mouse and human dystrophin (approximately 90%),⁴⁹ revertant expression of dystrophin and constitutive expression of apodystrophins in the plasmid-injected *mdx* mice. In addition, the immune rejection observed in *mdx* mice seemed to exceed the response developed by the dystrophin-competent C57BL/10 mice.

Intramuscular injections with naked DNA encoding viral, bacterial or tumoral antigens have been extensively studied.⁵⁰ A first DNA vaccine with the HIV virus *env* and *rev* genes was tested in an escalating dose assay on

15 infected asymptomatic patients. It showed an increase of the immune response in some patients, but with no significant modification of the number of CD4 and CD8 lymphocytes.⁵¹ Another assay of naked DNA vaccination was performed in healthy volunteers with a *Plasmodium falciparum* gene-carrying plasmid, and which showed CTL response.⁵²

However, the induction of an immune response seems less efficient in humans compared with animals.⁵³ Advantages of the muscle route of administration such as prolonged transgene expression and resistance of the antigenic source to clearance by the generated Ab could impede vaccination.⁵³ Indeed, long-term expression may lead to a state of tolerance towards the transgene.²⁹

It is, however, impossible to consider mouse data as predictive of the immune response in human DMD patients. Depending on the type of mutation, some patients are able to express low levels of endogenous dystrophin, which might induce immune tolerance for the exogenous expressed human protein. About 40% of the DMD patients display small amounts of dystrophin-expressing muscle fibers (less than 1%). More than 20% of patients are found to express low quantities of dystrophin. Those proportions probably underestimate the reality as 'false-negative fibers' may not be excluded depending the deletion or sensitivity of the protein detection protocols. It is also possible that certain forms of the dystrophin protein may not be properly located and detected. In Becker dystrophy, dystrophin expression is heterogeneous: low to strong expression of an often truncated dystrophin is observed in the muscle fibers.⁵⁴ Dystrophin isoforms which are often constitutively expressed by DMD patients may at least partially protect against immune rejection of an exogenous dystrophin. A phase I clinical trial for dystrophin gene transfer into DMD patients should therefore address the anti-dystrophin rejection issue (see also Ref. 25). In this respect, plasmid DNA may be a vector of choice for repeated injections, insensitive to the patient's pre-existing immunity and with limited risks compared with viruses and which allows long-term expression.²⁸

We have designed a phase I clinical trial using a full-length dystrophin plasmid to evaluate the potential immune response directed against an exogenous dystrophin, and at doses which have been already used in vaccination studies.^{49,52} The outcome of the study will have implications for future human dystrophin-gene trials: should an anti-dystrophin response occur, an adapted immunosuppressive should be envisaged, similar to those used in myoblast graft studies.^{14,17} Other approaches may potentially overcome the immune problem; this includes up-regulation of utrophin, an autosomal homologue of dystrophin, which could be able to replace dystrophin in DMD without risk of immune rejection as is constitutively expressed in DMD patients.⁵⁵ The perspectives for applications of i.m. delivery of plasmid DNA to DMD are hampered by its overall low efficiency. Nevertheless, direct intramuscular administration could be envisaged to treat small (for example, finger) muscles, as such, or after optimization of transfection including electroporation techniques. Furthermore, there is a potential for delivery of naked DNA to large muscle territories using locoregional intra-arterial administration⁵⁶ provided the immune aspect is well mastered.

Materials and methods

Vector constructs

Full-length human dystrophin plasmid: pTG11025 is an *E. coli* plasmid, based on the ColE1 origin of replication, and contains the kanamycin resistance gene encoded by *Tn903*. It carries an expression cassette encoding the full-length dystrophin cDNA driven by the human cytomegalovirus IE1 promoter (from pREP4, InVitrogen, Abingdon, UK). The mouse HMGC intron⁵⁷ and a SV40 polyadenylation signal are present immediately upstream and downstream of the dystrophin sequence. The backbone plasmid was named pTG11022 and used for *in vitro* transfections. The full-length dystrophin cDNA was generated,^{8,69} and kindly provided by Professor George Dickson (Royal Holloway, University of London, UK). Plasmid DNA was amplified in *E. coli* strain DH10B (Life Technologies, Cergy Pontoise, France) by overnight culture in LB medium (OD₆₀₀ = 2.5) and purified either for *in vitro* studies by double centrifugation on CsCl gradient as previously described,⁵⁸ or for *in vivo* use by an in-house protocol based on alkaline lysis, followed by a combination of ultrafiltration and gel filtration. Endotoxins were removed by liquid/liquid extraction with Triton X-114 according to a protocol derived from Bordier⁵⁹ and Manthorpe *et al.*⁶⁰

Purified plasmid DNA was concentrated to 1 mg/ml in either 10 mM Tris, 1 mM EDTA, pH 7.5 for *in vitro* transfections or in PBS or 0.9% NaCl for *in vivo* studies.

Adenovirus: Recombinant E1/E3 deleted human mini-dystrophin adenovirus under the control of the RSV promoter was obtained from Dr Perricaudet (UMR 1582, Villejuif, France).⁶¹

Preparation of antigens

Cell cultures: Dog primary myoblast cultures were obtained from skeletal muscle biopsies by explantation as described.^{35,62} Cells were expanded in Ham's F14 medium (Life Technologies) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 10 µg/ml insulin, 10 ng/ml EGF, 2 mM glutamine (all from Sigma-Aldrich, Saint Quentin Fallavier, France), 10 ng/ml bFGF (Pepro Tech, Rocky Hill, NJ, USA), and 40 µg/ml gentamycin (Schering-Plough, Kenilworth, NJ, USA) on gelatin-coated dishes.

Preparation of cellular extracts for Ab detection: Transfection was performed by calcium phosphate precipitation.⁶³ GRMD cells were seeded at 1.5×10^4 cells/cm² in 100-mm dishes 48 h before transfection. Precipitates were obtained at 50 µg plasmid/ml after 30 min precipitation and 100 µg plasmid per dish were added to cell layers for 16 h. Transfection was stopped by washing cells twice with Hank's balanced salts (Life Technologies) and renewing the culture medium. Cells were collected by trypsinization and washed in PBS/Complete 1X (antiproteases, Roche Diagnostics, Meylan, France) 24 h after initiation of transfection.

Protein solubilization: Proteins were solubilized in extraction buffer (75 mM Tris, 20% glycerol, 15% SDS, 100 mM

DTT, Complete 1X, pH 6.8), heated at 100°C for 3 min and passed through a 0.9-mm needle to shear DNA. The soluble fraction was collected after centrifugation for 5 min at 10 000 g and stored at -80°C. Protein concentration was determined according to Pande and Murthy.⁶⁴

In vivo gene transfer

C57BL/10 and *mdx* mice were bred in the Specific Pathogen-Free animal facility at Transgène S.A. Experiments were carried out on 4- to 8-week-old male mice. For i.m. administration, vectors were injected percutaneously in tibialis anterior (TA) muscles. Muscle regeneration was induced by i.m. administration of 3 ng notexin (Sigma-Aldrich) in each TA muscle 3 days before injection of the vectors. For intravenous (i.v.) administration, mice received a single injection of vector in the tail vein.

After various periods of time, mice were killed and TA muscles, blood and lymphoid organs (see below) were collected and prepared for immunohistochemistry.

Dystrophin immunohistochemistry

Muscle samples were embedded in OCT frozen in liquid nitrogen-cooled isopentane and stored at -80°C. Immunofluorescence microscopy using anti-dystrophin Ab was performed as follows: serial 8 µm cryostat transverse sections of unfixed muscles were prepared on glass slides and dipped in PBS buffer with 1% mouse serum, 30 min at room temperature for saturation of the non-specific binding sites. After rinsing (three times, 5 min) in PBS buffer, slides were dipped in PBS buffer containing 1/500 dilution of anti-dystrophin monoclonal Ab (NCL-DYS3 (Novocastra Laboratories, Newcastle, UK), MANDRA1 or MANDYS8 (both from Sigma-Aldrich) specific, respectively, for the N-terminal, C-terminal or central part of dystrophin) for 1.5 h at room temperature. Slides were rinsed three times 5 min in PBS, then incubated 30 min at room temperature with biotin-F(ab')₂ goat anti-mouse IgG (H+L; Immunotech, Marseille, France) diluted 1/500 in PBS. After rinsing (three times, 5 min in PBS), preparations were incubated 30 min at room temperature with 1/2000 streptavidin-FITC (Caltag Laboratories, Burlingame, CA, USA). Slices were then rinsed and mounted with Mowiol (Calbiochem/Novabiochem, La Jolla, CA, USA) and observed under an epifluorescence microscope, 200 magnification (Eclipse 800; Nikon, Tokyo, Japan).

Evaluation of the immune response

Detection of anti-human dystrophin Ab: Samples were collected at various time-points following vector administration, blood samples were collected from mice either by retro-orbital or cardiac puncture when animals were killed. Sera were collected after coagulation for 2-3 h at 4°C and centrifugation at 10 000 g for 2 × 10 min.

Protein samples were separated by SDS-PAGE on linear gradient of 4-12% polyacrylamide precast gels (15 wells, Novex, San Diego, CA, USA) and transferred on to nitrocellulose sheets (8 × 8 cm) for 5 h at 50 V in 50 mM Tris, 380 mM glycine, 0.01% SDS using a liquid-transfer apparatus. The membrane was blocked with 5% nonfat dry milk in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 8) for 45 min. Two-well strips were cut and incubated for 1.5 h with serum or anti-dystrophin mono-

clonal Ab (NCL-DYS1, Novocastra) diluted at 1/50 and 1/200, respectively, in 5% milk/TBST (1 ml per strip). After extensive washing for 3×1 min and 3×15 min with TBST (10 ml/strip), strips were probed for 1 h with peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Trappes, France) diluted at 1/5000 in 5% milk/TBST (5 ml/strip) and washed again. Ab-antigen complexes were detected using the ECL chemiluminescence system from Amersham Pharmacia Biotech (Orsay, France).

Hematoxylin-eosin staining: Cryosections were rehydrated and stained with hematoxylin-eosin (Merck, Darmstadt, Germany) according to the manufacturer's recommendations. Evaluation was performed at the Centre de Pathologie, Strasbourg by Dr Bernard Louis, anatomocytopathologist.

ELISpot (T cell activity)

Spleens and lymph nodes draining the injected muscle were removed sterilely and placed into tissue culture medium. Single cell suspensions were prepared and cells were frozen until use in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 20% fetal bovine serum and 10% dimethyl sulfoxide.

Preparation of human dystrophin-expressing mdx mouse lymphocytes: It was necessary for the purpose of the study to generate a vector expressing full-length dystrophin that is able to transduce antigen presenting cells (APC) efficiently. We have therefore constructed and produced a vaccinia virus vector.

Construction and production of full-length human dystrophin MVA vector (MVATG-14212) (details in manuscript in preparation): MVA strain was derived from Vaccinia virus Ankara strain by over 500 passages in primary chick embryo fibroblasts after which it could not replicate efficiently in a variety of mammalian cell lines.^{65,66} Our backbone vector (named MVATGN33.1) was isolated from the original material provided by Professor A Mayr in GMP conditions after two rounds of plaque purification.

Plasmid transfer: The transfer vector is based on plasmid pML2,⁶⁷ which was derived from pBR322. A *EcoRI* linker was inserted into pML2 leading to plasmid pTG1E which therefore contains a single *EcoRI* site, an *E. coli* origin of replication and the β -lactamase gene conferring ampicillin resistance. Flanking sequences surrounding the so-called deletion III of MVA were amplified by PCR from MVAN33.1 viral DNA and inserted within the *EcoRI* site of pTG1E (pTG6019). pTG6019 was modified by insertion within the *EcoRI* site of an expression cassette coding for the selection marker used for viral selection, the *Escherichia coli gpt* gene, placed under the control of the early-late promoter pH5R, for the isolation of recombinant MVA.⁶⁸ Synthesis of xanthine-guanine phosphoribosyltransferase enables *gpt*⁺ recombinant MVA to form plaques in a selective medium containing mycophenolic acid, xanthine, and hypoxanthine. The *gpt* gene was isolated from the plasmid pIV116 (a generous gift of Dr R Drillien, INSERM 99-08, Centre de Transfusion Sanguine, Strasbourg, France). Downstream of the selection marker expression cassette we have inserted a rep-

etition of the deletion III sequence homologous to the sequence upstream of the selection block. This type of construct would allow the natural excision of the selection block in the absence of a selection pressure. Moreover this construct (pTG14084) is completed by the insertion of a strong synthetic promoter named p11k75 kindly provided by R Wittek (Institut de Biologie Animale, Lausanne, Switzerland). pTG14084 was modified by insertion of a linker containing the unique restriction *NheI* flanked by the 5' end and 3' end of the human dystrophin cDNA. Human dystrophin cDNA was isolated from pTG11025 by a double digest with *NdeI/SalI* and inserted by homologous recombination within the pTG14207 linearized by *NheI* downstream the synthetic promoter p11k75. The resulting construct was named pTG14212.

Viral selection: pTG14212 was transfected within the MVA genome according to the standard calcium phosphate DNA precipitation procedure on to chicken embryo fibroblasts previously infected with MVATGN 33.1 at a MOI of 0.01 p.f.u. per cells. Viral selection was performed by three rounds of plaque purification in the presence of a selective medium containing mycophenolic acid, xanthine, and hypoxanthine on to chicken embryo fibroblasts. Absence of contamination by parental MVA was verified by PCR.

ELISpot assay: 96-well 'Multiscreen' ELISpot plates (Millipore, Bedford, MA, USA) were first coated with a monoclonal rat Ab specific for mouse interferon γ (Pharmingen, San Diego, CA, USA) at 3 μ g/ml overnight at 4°C. Wells were washed five times before the addition of cells and saturated with complete tissue culture medium.

To present antigen to the murine lymphocytes, EL4 cells were used. EL4 is a thymoma cell line from C57/BL6 mice which are H-2b and therefore histocompatible with *mdx* mice. Antigen presenting EL4 cells were taken from log phase growth culture and infected, at a multiplicity of infection of 1.0, with MVA-N33 (no insert) or MVATG-14212 (human dystrophin). Five million cells were infected in a volume of 200 μ l PBS (containing cations) supplemented with 1% FBS. Virus was allowed to adsorb for 1 h at room temperature, then diluted in 30 ml of complete culture medium (DMEM + 10% FCS) and transferred to a Falcon F75 flask. The following day, infected EL4 cells were suspended in DMEM supplemented with 10% FCS and 50 μ g/ml Mitomycin-C (Sigma) in the dark, at 37°C for 1 h. Treated, infected cells were then washed six times with DMEM. Cells were then counted and added to wells of the ELISpot plate at 5×10^4 per well (experiment 1) or 10^5 per well (experiment 2). Data are expressed as mean $\pm$ s.e.m. of four determinations (four wells per individual measurement). Cellular response is considered positive when differences between negative control antigen (MVA-N33 with no insert) and test antigen (MVATG-14212 carrying human dystrophin) exceed 3 s.e.m.

Spleen and lymph node cells were thawed and counted. Spleen cells were centrifuged over Lympholyte-M (Cedarlane, Hornby, Canada) density centrifugation medium. Viable cells from the interface were washed in culture medium and counted. Viable spleen cells were distributed into the ELISpot wells at 5×10^5 cells per well. To each well, 30 IU human IL-2 (Roche Diagnostics, Mey-

lan, France) was added. Plates were then cultured for 2 days at 37°C, 5% CO₂. At the end of the 2-day culture period, cells were removed by six washes with PBS + 0.05% Tween 20. 100 µl per well of biotinylated rat anti-mouse IFNγ (Pharmingen, San Diego, CA, USA) at a concentration of 3 µg/ml were added and incubation pursued at room temperature for 2 h. Plates were then washed five times and 100 µl per well of extravidin phosphates (Sigma-Aldrich) was added at a dilution of 1/5000 and set to react at room temperature for 45 min. Plates were then washed five times and enzyme activity revealed with the Alkaline Phosphates Conjugate Substrate kit (BioRad, Hercules, CA, USA) for 20 min. Reaction was stopped by abundant washing with tap water. Spots were then counted using a binocular microscope.

Theoretically, after APC are infected and washed, there are no remaining infectious particles. We have been concerned that infectious viral particles may remain attached to the APC without infecting the APC. This would mean that infectious particles could be liberated from the APC and, in turn, infect the T cells which are eventually added. This could result in the stimulation of IFNγ release by T cells due to infection by the virus rather than by recognition by the viral or recombinant antigens by the T cells. We have tried eliminating the remaining infectious particles by treatment with UV light. This treatment eliminates infectious particles, but it also drastically decreases antigen presenting capacity of the cells. We also tested the ability of MVA to infect non-adherent lymphoid cells which would result in the production of IFNγ. We found that an MOI of at least 10 is required. Thus, for 10⁵ lymphoid cells, 10⁶ to 10⁷ p.f.u. would be required. 1000 p.f.u. resulted in no detectable ELISpots. A maximum of 1000 p.f.u. has been found to remain attached to 10⁵ APC after infection. Therefore, ELISpots cannot be attributed to infection of T cells as opposed to immune recognition of antigens (data not shown).

Anti-DNA Ab

Mouse sera were tested for the presence of anti-DNA Ab using an ELISA assay based on commercial 12-wells dsDNA-coated polystyrene strips (The Binding Site, Birmingham, UK) initially designed for human clinical usage. As a positive control, a commercial mouse anti-DNA monoclonal Ab (Pierce, Rockford, IL, USA) was used. A peroxidase-conjugated anti-mouse Ab was used for colorimetric detection. The assay ranged from 3.0 µg/ml to 750 µg/ml of specific anti-DNA Ab.

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