FEBS Letters 407 (1997) 164–168 FEBS 18474

Immune responses, not promoter inactivation, are responsible for decreased long-term expression following plasmid gene transfer into skeletal muscle

K.E. Wells[a], J. Maule[a], R. Kingston[a], K. Foster[a], J. McMahon[a], E. Damien[b], A. Poole[b], D.J. Wells[a,*]

[a] *Gene Targeting Unit, Departments of Pharmacology and Clinical Neuroscience, Charing Cross and Westminster Medical School, St. Dunstan's Road, London W6 8RF, UK*

[b] *Department of Veterinary Basic Sciences, The Royal Veterinary College, London NW1 OTU, UK*

Received 4 March 1997

Abstract Long-term high-level in vivo gene expression appears to depend on the promoter chosen to drive the gene of choice. In many cases the promoter appears to 'switch off' some time after in vivo gene transfer. We demonstrate that, following intramuscular injection of β-galactosidase reporter plasmids, promoter 'switch off' is due to elimination of fibres expressing the transferred reporter gene by activation of a Th1 (cytotoxic) immune response. This finding, in the absence of stimulation of the immune system by viral vector proteins, has implications not only for gene transfer experiments but for the future of muscle-directed gene therapy.

Key words: Gene therapy; Plasmid DNA; Skeletal muscle; Cytotoxic T-cells; Immunology

1. Introduction

Skeletal muscle is an excellent target for in vivo gene transfer. Once genetic material is introduced into muscle fibres it can remain there for very many months and may last for life (e.g. mouse studies [1]). This longevity of expression is probably due to the multinucleate post-mitotic nature of differentiated skeletal muscle and reflects the stability of these structures. Consequently, skeletal muscle has been considered to serve as a useful platform for systemic expression of gene products in the treatment of non-muscle diseases, as well as being the target for direct genetic treatment of diseases such as Duchenne muscular dystrophy. Such gene therapies will require promoters that drive high-level expression in muscle. Viral promoters produce much higher levels of expression than eukaryotic promoters and one of the most widely used promoter constructs is derived from the cytomegalovirus (CMV) intermediate early promoter and enhancer. It has been reported that this promoter appears to decline in activity over time [2,3]. We examined the activity of this CMV promoter and the Rous sarcoma virus (RSV) promoter in normal and immunocompromised animals to determine if this decline in expression was a property of the promoter or an in vivo response to foreign gene expression.

2. Materials and methods

2.1. Plasmid production

The following three plasmids were used in the experiment: pCMV-β, which utilises the CMV intermediate early promoter and enhancer to drive the bacterial β-galactosidase reporter gene (Clontech, Palo Alto, CA, USA); pRSV-β, which utilises the RSV intermediate early promoter to drive the bacterial β-galactosidase reporter gene [4]; pCMV-0, which was made from pCMV-β by removing the β-galactosidase reporter gene with a *Not*I digest and religating the plasmid backbone. Plasmids were grown in JM109 *E. coli* and purified using Qiagen columns (Diagen) following the supplier's protocol with the following exception: after the final clearing spin, but before application to the column, the lysate was incubated on ice for 5 min with Triton X-114 (final concentration 1%) to reduce endotoxin contaminants [5]. This step typically reduces endotoxin levels to less than 0.5 IU/μg of plasmid, as assessed using the *Limulus* amoebocyte lysate assay (Etoxate, Sigma). The cleared treated lysate was then applied to the column and rinsed with 1.5 times the manufacturer's recommended wash buffer volume. The rest of the procedure followed the manufacturer's instructions. Both restriction enzyme-cut and uncut plasmids were checked by agarose gel electrophoresis to confirm identity. This analysis demonstrated the absence of detectable contamination with RNA and that the majority (90%) of each plasmid was present as covalently closed circles.

2.2. Injection of plasmid DNA in vivo

BALB/c and nude BALB/c male mice were obtained from Harlan Olac, UK. They were reared in a minimal disease environment with food and water available ad libitum. Seven-week-old mice were injected percutaneously with 25 μl of a 1.2% solution of barium chloride in normal saline, using a 27 gauge needle, into the middle of both anterior tibial muscles under Hypnorm-Hypnovel general anaesthesia [6]. Five days later, 25 μg of plasmid DNA in normal saline at 1 μg/μl was similarly injected into the middle of each pre-treated muscle, again under general anaesthesia. Blood samples were taken by tail bleeding prior to injection of plasmid and at weekly intervals thereafter up to 6 weeks post injection of plasmid DNA. Pre-treatment with barium chloride was used to induce muscle regeneration as such regeneration has previously been shown to dramatically enhance gene expression following plasmid DNA gene transfer [7–10]. Barium chloride is a particularly effective myotoxic agent in mice giving reproducibly high levels of muscle regeneration [11]. A similar protocol was followed for mice used in the transfection, histochemistry and cytotoxic T lymphocyte (CTL) assay experiments but samples were taken at different time points as detailed below.

2.3. Histochemistry and immunohistochemistry

At 1 week post plasmid injection 6 mice in each treatment group were killed with an overdose of barbiturate anaesthetic followed by cardiac puncture to collect blood. The anterior tibial muscles were dissected free from overlying connective tissue. One muscle of each pair was frozen in liquid nitrogen as a reserve and the other muscle was mounted on a cork block, covered in embedding compound (Cryo-M-Bed, Bright, UK) and flash-frozen in isopentane cooled in liquid nitrogen. The same procedure was carried out for 6 animals in

*Corresponding author. Fax: (44) (181) 846-7377.

PII S0014-5793(97)00329-3

each treatment group at 6 weeks post plasmid injection. Serial transverse sections were cut at 10 μm in a cryostat from 10 equally spaced sites along the length of the muscle, were fixed in paraformaldehyde and then stained in a solution of 1 mM X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM magnesium chloride in PBS for 1 or 24 h. After washing in PBS, the slides were counterstained in alcoholic eosin, dehydrated in ethanol and mounted in DPX. Other acetone-fixed slides were stained with a rat monoclonal antibody directed against mouse CD4 or CD8 [12], using rabbit anti-rat immunoglobulin G (IgG) as the secondary antibody and visualised with Streptavidin-ABC and DAB followed by counterstaining for β-galactosidase. Fibre counts were analysed statistically by two-way ANOVA and Student-Newman-Keuls multiple paired comparisons.

2.4. β-Galactosidase and chloramphenicol acetyltransferase (CAT) ELISAs

For analysis of total β-galactosidase expression, 7 BALB/c mice were pre-treated with barium chloride as above. Five days following pretreatment, 25 μg of CMV-β was injected into the left anterior tibial muscle and 25 μg of RSV-β was injected into the right. In both cases, plasmids were coinjected with 25 μg of RSV-CAT to control for transfection efficiency. Muscles were collected 1 week post plasmid injection and flash-frozen in liquid nitrogen. 100 μl of lysis buffer (from Boehringer β-galactosidase or CAT ELISA kit) was added with a cocktail of protease inhibitors (50 μg/ml antipain, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin and 2 μl/ml of a 10 mg/ml stock of PMSF in ethanol). The tissue was homogenised with a micropestle and subjected to four freeze/thaw cycles. A further 400 μl of lysis buffer with protease inhibitor was added, mixed thoroughly and then spun at 13000 rpm for 10 min to pellet all insoluble material. Supernatants were stored at −70°C until assayed. For each assay 50 μl of supernatant was diluted to 200 μl and assayed using the appropriate Boehringer kit as per the manufacturer's instructions. CAT enzyme levels were used to normalise the β-galactosidase results to compensate for variation in transfection efficiency. Transfection data were analysed statistically with the Wilcoxon signed rank test.

2.5. Antibody ELISA

Microtitre plates (Highbind Multibind flatwell, Grenier, UK) were coated with *E. coli* β-galactosidase (Sigma) at 10 μg/ml in carbonate buffer the day prior to using the plates. Coated plates were blocked with a 3% solution of milk powder prior to incubation with serial dilutions of mouse serum. A mouse monoclonal anti-β-galactosidase (Sigma G 8021) was used as a positive control. After washing three times with PBS (including 0.05% Tween) the secondary antibody, rabbit anti-mouse IgG horseradish peroxidase (Biorad), was applied for 30 min at 37°C at a dilution of 1:4000 in PBS with 1% FCS. Binding of the secondary antibody was visualised with the OPD reaction developed for 30 min at room temperature and the optical density of each well was determined at 492 nm using a plate reader. A panel of rabbit polyclonal mouse isotype-specific secondary antibodies (Z0013-Z0016, Dako, UK) were used to identify the IgG isotype response in responding animals using the above procedure. Data are presented after subtraction of background reading from duplicate wells not coated with β-galactosidase.

2.6. Cytotoxicity assays

Animals were treated as detailed in Section 2.2. Spleens were harvested at 10 weeks post plasmid injection and cells collected into Glutamax/pyruvate DMEM, 50 μM 2ME, 10% fetal calf serum (FCS), 1% non-essential amino acids. After a brief period to settle clumps, red blood cells were lysed with 5 ml ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA at pH 7.2) per spleen for 5 min at room temperature, cells were washed once in PBS and trypan blue-excluded viable cell counts were taken. P815 cells were treated with mitomycin C for 20 min at 37°C (50 μg/ml for 1×10^7 cells/ml), washed thoroughly and then pulsed for 1 h at 37°C with 10 μM of the β-galactosidase H-2L^d-restricted cytotoxic epitope peptide (TPHPARIGL [13]). Splenocytes were cocultured at 37°C in 5% CO_2 with peptide-pulsed mitomycin C-treated P815 cells at an effector to stimulator ratio of 1:1 in the presence of 10 U/ml of human recombinant IL-2 (Sigma) at an initial cell concentration of 10^6 cells/ml. Cultures were harvested after 5 days and viable cells recovered from Nycoprep gradients (Nycoprep (Animal) 1.077, Nycomed). The CTL assay was carried out with 4 h incubation plus or minus 10^4 P815 target cells per well in a 96-well round-bottomed plate with or without the addition of 10 μM TPHPARIGL peptide. FCS levels were dropped to 5% for the assay to reduce background LDH levels. The assay was performed using the Promega Cytox Assay kit as per manufacturer's instructions.

3. Results

3.1. β-Galactosidase expression in muscle

Fig. 1 shows the number of β-galactosidase-positive fibres in mouse anterior tibial muscles at 1 and 6 weeks post plasmid injection. No β-galactosidase-positive fibres were seen in muscles injected with the CMV-0 construct. Relatively few positive fibres were detected in RSV-β-injected muscles. This number declined between 1 and 6 weeks but the difference is not significant. The muscles injected with CMV-β plasmid showed a large number of fibres expressing β-galactosidase at 1 week post injection, but this number had fallen significantly to very low levels by 6 weeks post injection ($P < 0.05$). Nude animals injected with CMV-β showed a variable but generally high number of β-galactosidase-positive fibres at

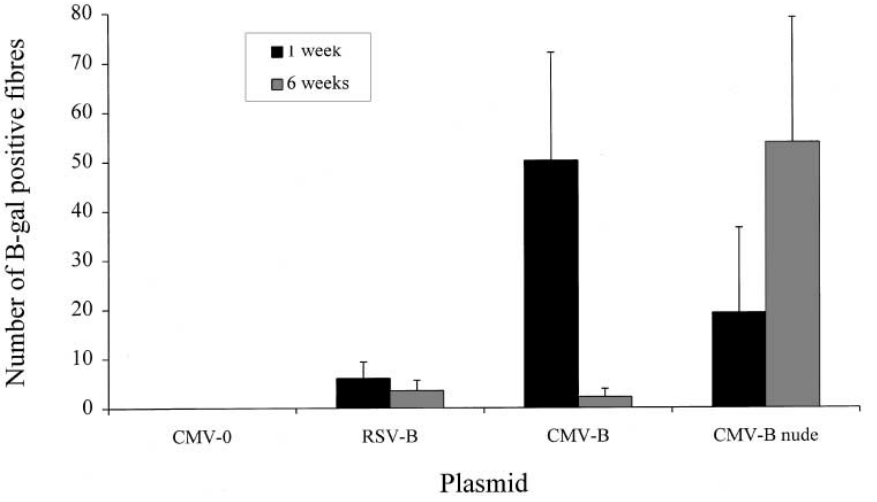

Fig. 1. The mean number of β-galactosidase-positive fibres for each plasmid treatment at 1 week (black bars) and 6 weeks (grey bars) post injection. T-bars display the standard error of the mean with $n=6$ in the first three groups and $n=5$ in the nude group.

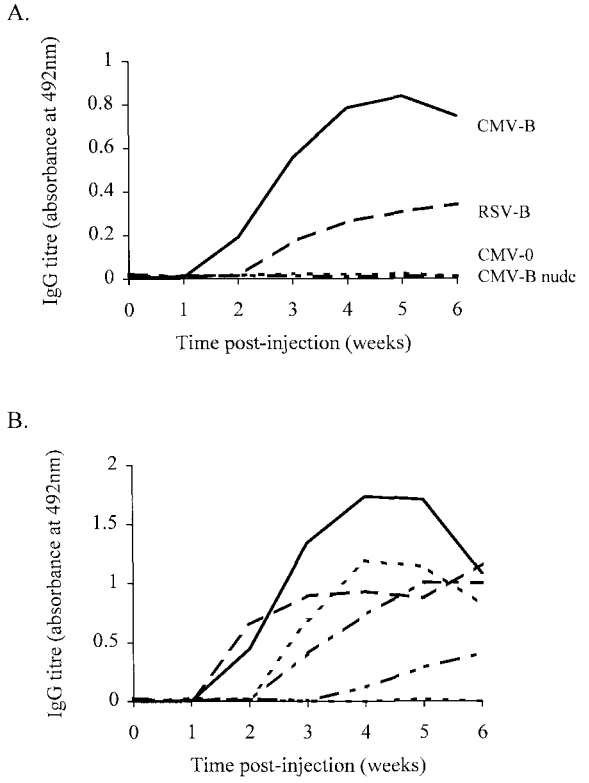

Fig. 2. A: Mean value for total IgG titre detected at a serum dilution of 1:100 for each weekly blood sample of animals injected with CMV-β (solid line), RSV-β (dashed line), CMV-0 (dotted line) and nude mice injected with CMV-β (dot-dash line). Only the first two treatments produced titres above baseline. B: Individual animal total IgG titres detected at a serum dilution of 1:100 for each weekly blood sample of animals injected with CMV-β. Considerable variation between individuals in evident with one animal showing no response.

1 week post injection and this number did not decline over the 6 week period of the experiment. The considerable variation in number of positive fibres when comparing animals is typical of such experiments [2,14]. The transfection assay with CMV-β gave a CAT adjusted average of 12.74 ± 6.69 S.E. ($n = 7$) compared to 0.798 ± 0.067 S.E. ($n = 7$) for RSV-β, a significant 16-fold difference in levels of expression between the two promoters at 1 week post plasmid injection ($P = 0.03$).

3.2. *Immune responses to β-galactosidase*

Fig. 2A shows the total IgG response to β-galactosidase over the 6 week period. Not surprisingly, neither the nude animals injected with CMV-β nor the normal animals injected with CMV-0 showed any response to β-galactosidase above the normal background. All but one CMV-β-injected animal demonstrated significant antibody titres to β-galactosidase although there was considerable variation between animals (Fig. 2B). Animals injected with the RSV-β plasmid showed lower anti-β-galactosidase antibody levels compared to those injected with CMV-β. Subtyping the IgG response in the normal animals injected with the CMV-β plasmid clearly demonstrates a predominantly IgG_{2a} response (Fig. 3) but those animals demonstrating a humoral immune response following injection with RSV-β had a lower IgG_{2a} to IgG_{2b} ratio. IgG_1 titres were as high as those for IgG_{2b} in the CMV-β injected animals and there was also a significant IgG_3 titre. In contrast the IgG_1 titre was much lower than the IgG_{2b} and there was no detectable IgG_3 response in the RSV-β injected animals.

The CTL assay demonstrated a strong specific lysis of the peptide-pulsed P815 target cells and minimal non-specific lysis by splenocytes from CMV-β treated animals (Fig. 4). In contrast splenocytes from CMV-0-treated animals showed low but similar lysis of both peptide- and non-peptide-pulsed P815 target cells. This result clearly demonstrates a β-galactosidase-specific CTL response in those animals injected with the β-galactosidase-expressing CMV-β plasmid.

3.3. *Immunohistochemistry*

Sections from samples taken at 1 week post injection with all plasmids showed a heavy mononuclear cell infiltrate containing some CD4-positive cells and few CD8-positive cells. By 2 weeks muscles injected with the CMV-β plasmid show β-

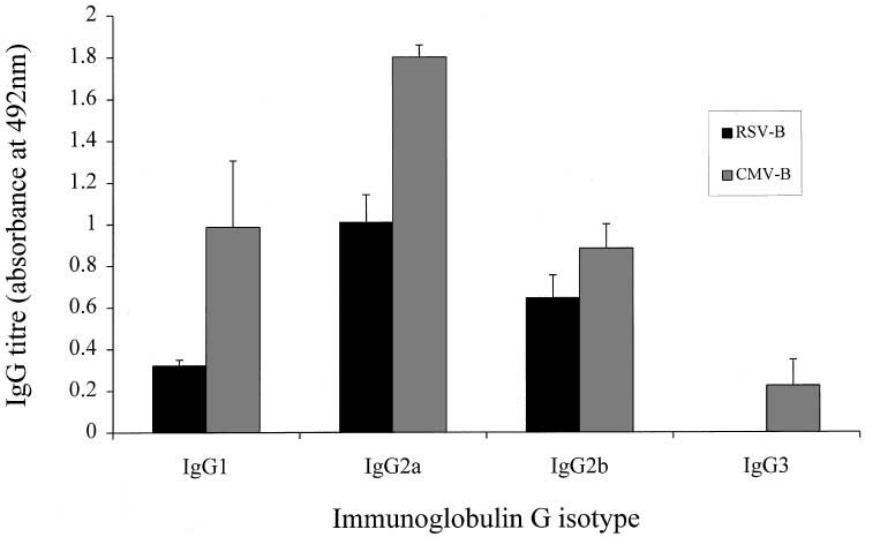

Fig. 3. The mean IgG titres at a serum dilution of 1:100 for each subclass at 6 weeks post injection for animals showing a humoral immune response to β-galactosidase following injection of RSV-β ($n = 3$, black bars) and CMV-β ($n = 5$, grey bars). T-bars display the standard error of the mean.

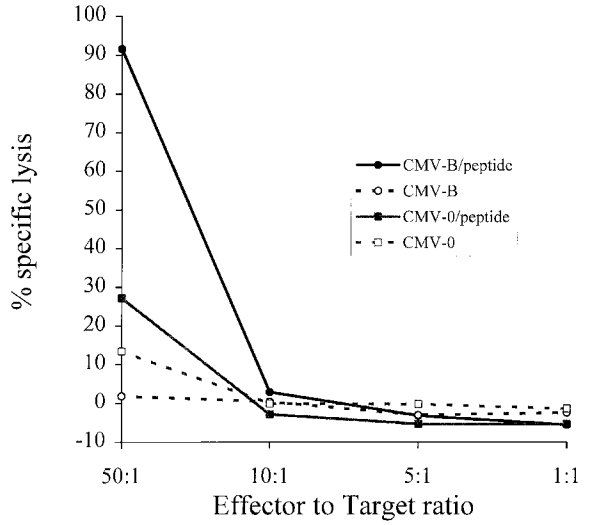

Fig. 4. The corrected percentage maximum specific lysis generated by splenocytes from CMV-β-treated animals (circles) and CMV-0-treated animals (squares) at a variety of effector to target ratios. Target P815 cells were pulsed with β-galactosidase-specific peptide (solid lines) or left untreated (dashed lines).

galactosidase-positive fibres apparently under attack by mononuclear cells, many of which stain positively for the CD8 cell surface marker, indicating they are cytotoxic T-cells (Fig. 5). Samples taken at 6 weeks post injection have a minimal mononuclear cell infiltrate in the muscle sections.

4. Discussion

The continued presence of high numbers of β-galactosidase-positive fibres in the nude animals injected with CMV-β plasmid is evidence that this loss of positive fibres is not due to the inactivation of the CMV promoter, as previously postulated [2,3]. Indeed, the substantial antibody response to β-galactosidase, the specific in vitro CTL assay and the presence of $CD8^+$ mononuclear cells around β-galactosidase-positive fibres strongly suggests that the loss of β-galactosidase-positive fibres in the RSV-β- and CMV-β-injected normal mice is due to the action of the immune system. The predominant IgG_{2a} response points to activation of the immune system via the Th1 pathway and this pathway is responsible for the activation of cytotoxic T lymphocytes [15]. Much of the difference in the immune system response to CMV-β compared to RSV-β can be attributed to the level of expression of the β-galactosidase, as demonstrated by the high number of β-galactosidase-positive fibres at 1 week following injection of CMV-β (×8) and the overall higher level of gene expression seen in the transfection assay (×16). However, the relative differences in isotypes comparing RSV-β-injected animals to those treated with CMV-β raises the possibility that there may be differences in the transient expression in non-muscle cells including professional antigen presenting cells. The CMV promoter directs ubiquitous expression [16] whereas the RSV promoter appears to express particularly well in muscle [17]. Such differences could help to explain why long-term gene expression has been observed in non-nude animals using the RSV promoter and a less strongly antigenic reporter gene, luciferase [1]. Interestingly, the isotype profiles from these experiments in muscle are different from very similar experiments examining gene transfer into skin [18]. In the skin studies, the ratio of IgG_{2a} to IgG_1 antibodies was very much higher than in this muscle study and may reflect a lack of destruction of the skin cells [19], and hence less antigen release, compared to the response to gene expression in muscle. Alternatively, skeletal muscle may secrete substantial amounts of β-galactosidase compared to skin cells.

We believe that immune responses to exogenous proteins produced in muscle following somatic gene transfer can lead to the loss of transfected fibres over time. This fibre loss places an important potential limitation on the long-term success of gene transfer strategies. Although β-galactosidase is recognised as a powerful immunogen (e.g. [20–23]), immune system responses have been reported to other gene products following gene transfer into muscle (e.g. [24–26]) and it seems quite probable that in many cases such immune responses may lead to a clearance of transfected fibres over time. In the studies cited [20–26], viral vectors were used to introduce the exogenous gene and the presence of viral proteins from the vector, or leakage of viral proteins from the replication-deficient genome of such vectors, may well have influenced the nature of the immune response that was recorded. In contrast, the plasmid gene transfer system does not introduce potentially immunogenic proteins at the time of gene transfer and

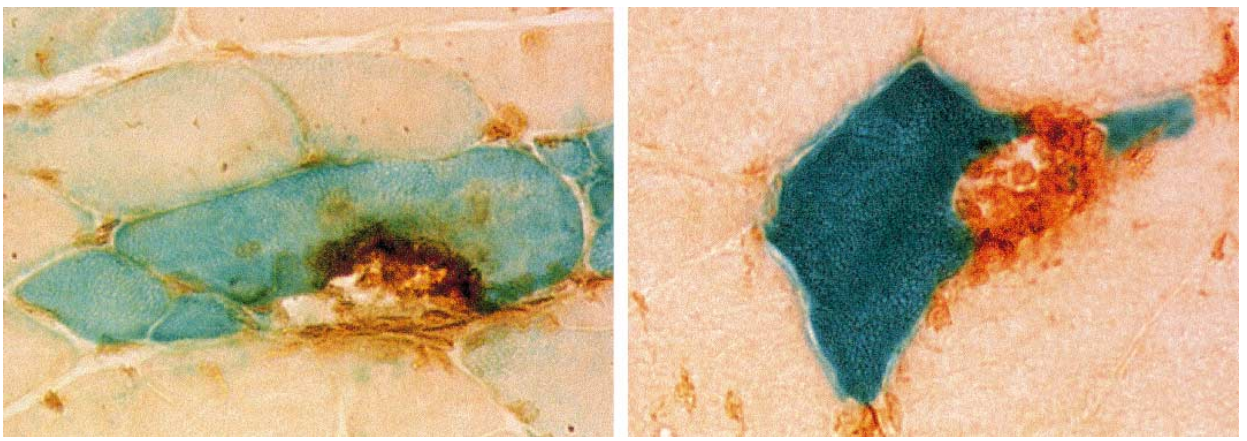

Fig. 5. Representative examples of muscle fibres in cross-section from animals injected with the CMV-β plasmid 2 weeks prior to sampling. The sections were immunostained for CD8-positive cells and then histochemically stained for β-galactosidase expression. Dark brown CD8-positive cells can be seen attacking the edges of blue-stained muscle fibres that are expressing β-galactosidase. Images are magnified 1700 times.

so results obtained can be specifically attributed to expression of the transferred gene. Our observations of the considerable loss of transfected fibres suggest the immune system could be a serious limitation to gene therapy strategies for diseases of muscles, such as Duchenne muscular dystrophy, and may limit the long-term systemic expression of proteins for both systemic treatment and vaccination strategies.

Acknowledgements: This work was supported by grants from the Medical Research Council of Great Britain and the Muscular Dystrophy Group of Great Britain and Northern Ireland. We thank Dr. Baker, Institute of Ophthalmology, for the gift of rat monoclonal antibodies to mouse CD4 and CD8. The β-galactosidase peptide was made by the Advanced Biotechnology Centre at Charing Cross and Westminster Medical School.

References

[1] J.A. Wolff, J.J. Ludtke, G. Acsadi, P. Williams, A. Jani, Hum Mol Genet 1 (1992) 363–369.
[2] M. Manthorpe, F. Cornefert-Jensen, J. Hartikka, J. Felgner, A. Rundell, M. Margalth, V.J. Dwarki, Hum Gene Ther 4 (1993) 419–421.
[3] J. Hartikka, M. Sawdey, F. Cornefert-Jensen, M. Margalith, K. Barnhart, M. Nolasco, H.L. Vahlsing, J. Meek, M. Marquet, P. Hobart, J. Norman, M. Manthorpe, Hum Gene Ther 7 (1996) 1205–1217.
[4] P.A. Norton, J.M. Coffin, Mol Cell Biol 5 (1985) 281–290.
[5] Y. Aida, M.J. Pabst, J Immunol Methods 132 (1990) 191–195.
[6] Flecknell PA. Laboratory Animal Anaesthesia. London: Academic Press, 1987.
[7] H.L. Davis, B.A. Demeneix, B. Quantin, J. Coulombe, R.G. Whalen, Hum Gene Ther 4 (1993) 733–740.
[8] D.J. Wells, FEBS Lett 332 (1993) 179–182.
[9] I. Danko, J.D. Fritz, S. Jiao, K. Hogan, J.S. Latendresse, J.A. Wolfe, Gene Ther 1 (1994) 114–121.
[10] M. Vitadello, M.V. Schiaffino, A. Picard, M. Scarpa, S. Schiaffino, Hum Gene Ther 5 (1994) 11–18.
[11] A. Fassati, D.J. Wells, F.S. Walsh, G. Dickson, Hum Gene Ther 6 (1995) 1177–1184.
[12] D. Baker, J.K. O'Neill, S.E. Gschmeissner, C.E. Wilcox, C. Butter, J.L. Turk, J Neuroimmunol 28 (1990) 261–270.
[13] M.A. Gavin, M.J. Gilbert, S.R. Riddell, P.D. Greenburg, M.J. Bevan, J Immunol 151 (1993) 3971–3980.
[14] J.A. Wolff, P. Williams, G. Acsadi, S.S. Jiao, A. Jani, C. Wang, BioTechniques 11 (1991) 474–485.
[15] N.E. Street, T.R. Mosmann, FASEB J 5 (1991) 171–177.
[16] M. Boshart, F. Weber, G. Jahn, K. Dorsch-Hasler, B. Fleckenstein, W. Schaffner, Cell 41 (1985) 521–530.
[17] P.A. Overbeed, S.P. Lai, K.R. Van-Quill, H. Westphal, Science 231 (1986) 1574–1577.
[18] E. Raz, H. Tighe, Y. Sato, M. Corr, J.A. Dudler, M. Roman, S.L. Swain, H.L. Spiegelberg, D.A. Carson, Proc Natl Acad Sci USA 93 (1996) 5141–5145.
[19] E. Raz, D.A. Carson, S.E. Parker, T.B. Parr, A.M. Abai, G. Aichinger, S.H. Gromkowski, M. Singh, D. Lew, M.A. Yankauckas, S.M. Baird, G.H. Rhodes, Proc Natl Acad Sci USA 91 (1994) 9519–9523.
[20] H. Gilgenkrantz, D. Duboc, V. Juillard, D. Couton, A. Pavirani, J.G. Guillet, P. Briand, A. Kahn, Hum Gene Ther 6 (1995) 1265–1274.
[21] F.W. Van Ginkel, C. Liu, J.W. Simecka, J.-Y. Dong, T. Greenway, R.A. Frizzell, H. Kiyono, J.R. McGhee, D.W. Pascual, Hum Gene Ther 6 (1995) 895–903.
[22] J.-T. Vilquin, B. Guerette, I. Kinoshita, B. Roy, M. Goulet, C. Gravel, R. Roy, J.P. Tremblay, Hum Gene Ther 6 (1995) 1391–1401.
[23] J.Y. Dong, D. Wang, F.W. Van Ginkel, D.W. Pascual, R.A. Frizzell, Hum Gene Ther 7 (1996) 319–331.
[24] Y. Dai, E.M. Schwarz, D. Gu, W.W. Zhang, N. Sarvetnick, I.M. Verma, Proc Natl Acad Sci USA 92 (1995) 1401–1405.
[25] K.F. Kozarsky, D.R. McKinley, L.L. Austin, S.E. Raper, L.D. Stratford-Perricaudet, J.M. Wilson, J Biol Chem 269 (1994) 13695–13702.
[26] S.K. Tripathy, H.B. Black, E. Goldwasser, J.M. Leiden, Nature Med 2 (1996) 545–550.