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***BRIEF COMMUNICATION***

# *Inflammatory responses following direct injection of plasmid DNA into skeletal muscle*

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*Transfer of genes by injection of plasmid DNA into skeletal muscle has a wide variety of applications ranging from treatment of neuromuscular disorders to genetic vaccination. We examined each component involved in the intramuscular injection of plasmid DNA in terms of the induction of inflammatory responses. The insertion of a needle and the injection of a relatively large volume of saline caused very little muscle damage except in rare cases. In contrast, barium chloride-induced regeneration of muscle, injection of lipopolysaccharide, plasmid backbone or plasmid expressing a neo-antigen (β-galactosidase) all generated widespread inflammation of injected muscle, with mononuclear infiltrate, comprised largely of macrophages and with both CD4+ and CD8+ T lymphocytes, present. Such inflammation may hamper clinical application of this technology and may encourage undesirable immune responses in gene therapy trials. Inflammation was not greatly reduced by CD4- or CD8-depleting antibodies, suggesting this initial inflammation did not involve T cells, but methylation of plasmid DNA before injection substantially lessened the inflammatory response and resulted in longer term expression of the transgene.*

**Keywords:** *gene therapy; regeneration; β-galactosidase; muscle; plasmid; inflammation*

Skeletal muscle has the ability to take up and express naked plasmid DNA following intramuscular injection[1,2] and hence is an attractive platform for (1) providing a systemic source for recombinant therapeutic proteins;[3,4] and (2) as a means of genetic vaccination against either bacteria and viruses,[5–7] or tumour cells.[8–10]

However, long-term expression of transgenes encoded in the plasmid DNA can be limited by the immune responses (cellular, humoral and innate) evoked during the processes of gene uptake and expression in skeletal muscle fibres.[11–15] These responses, while necessary and desirable for genetic vaccination, can seriously limit the use of direct plasmid injection for gene therapy. Immune responses also play a significant role in determining the success of virus-based gene transfer.[16–22] In order to start to dissect the relative roles of the responses to vector and transgene, we have concentrated on gene transfer by direct injection of plasmid DNA. This system does not involve injection of any exogenous protein component, allowing the characteristics of the antigen-specific response to be analysed separately. However, plasmid DNA is prepared in *E. coli* which results both in contamination with lipopolysaccharide (LPS, endotoxin) and the presence of unmethylated CpG motifs. LPS is a known stimulator of immune responses and is frequently used for *in vitro* stimulation of lymphocyte populations.[23–25] Unmethylated CpG motifs are comparatively rare in eukaryotic genomes[26] and hence immunostimulatory sequences (ISSs[27]) containing unmethylated CpGs, in particular sequence contexts, are probably recognised as a 'danger signal',[28] or as a pathogen-associated molecular pattern or PAMP.[29] These sequences invoke innate immune responses including activation of B cells and cytokine production from monocytes, whereas eukaryotic genomic DNA does not activate the immune system *in vivo*[30] or *in vitro*.[31,32] The presence of these sequences in plasmid vectors used for genetic vaccination have increased the immune response despite lower expression levels.[33]

In this study, we examined the various components of the direct injection procedure in order to assess their contribution to the resulting inflammatory response in both non-regenerated and regenerated muscle. We also attempted to modify this inflammatory response by administration of depleting antibodies or by methylating immunostimulatory CpG motifs in plasmid DNA before *in vivo* administration.

Six- to 8-week-old male C57/Bl10 mice were divided into two groups. One group received no pretreatment and the other group had muscle regeneration induced by injection of a barium chloride solution into both anterior tibial muscles. Animals from each group then received one of four different treatments to the left anterior tibial muscle. In each group, four mice received the same treatment.

Table 1 summarises the histological analysis of non-regenerated and regenerated muscle respectively, 7 days after injection with (1) needle alone; (2) normal saline; (3) a plasmid backbone, pBluescript (pBS; Stratagene, Cambridge, UK); or (4) a plasmid encoding a neo-antigen, CMVβ (Clontech, Palo Alto, CA, USA). When *in vivo* treatments were applied to non-regenerated muscle,

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**Table 1** Histological analysis of injected muscle

| *Number* | *Needle-stick only* | | | *Saline* | | | *pBluescript* | | | *CMVβ* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *CD4* | *CD8* | *H&E* | *CD4* | *CD8* | *H&E* | *CD4* | *CD8* | *H&E* | *CD4* | *CD8* | *H&E* |
| *(a) Non-regenerated muscle* | | | | | | | | | | | | |
| 1 | – | – | + | + | + | + | ++ | ++ | ++/+++ | +++ | ++ | +++ |
| 2[a] | ++ | ++++ | ++++ | +/++ | + | + | +++ | + | +++ | + | ++ | +++/++++ |
| 3 | + | – | + | + | + | + | + | ++ | ++ | + | ++ | +++ |
| 4 | – | + | + | + | + | + | + | ++ | ++/+++ | + | ++ | +++ |
| C1 | – | – | + | – | – | +/– | – | – | +/– | – | – | +/– |
| C2 | – | – | +/– | – | – | +/– | – | – | +/– | – | – | +/– |
| C3 | – | – | +/– | – | – | +/– | – | – | +/– | – | – | +/– |
| C4 | – | – | +/– | – | – | +/– | – | – | +/– | – | – | +/– |
| *(b) Regenerated muscle* | | | | | | | | | | | | |
| 1 | ++ | + | +++++ | ++/+++ | +/++ | +++/++++ | ++++ | +++ | +++++ | ++++ | ++++ | +++++ |
| 2 | + | + | +++ | ++ | + | ++/+++ | +++++ | ++++/+++++ | ++++ | +++/++++ | +++/++++ | +++++ |
| 3 | ++/+++ | +/++ | +++/++++ | ++ | +/++ | ++ | +++ | +++/++++ | +++/++++ | +++ | +++/++++ | +++++ |
| 4 | ++/+++ | +/++ | +++/++++ | ++ | +/++ | ++ | +++/++++ | ++++ | ++++/+++++ | ++++ | +++/++++ | +++++ |
| C1 | ++ | +/++ | ++++ | ++ | +/++ | +++/++++ | + | + | +++ | ++ | +/++ | ++++ |
| C2 | ++ | + | + | ++/+++ | +/++ | +++/++++ | + | + | +++ | +/++ | + | +++ |
| C3 | ++ | + | ++ | ++ | ++ | +++ | +++/++++ | + | +++++ | ++/+++ | ++/+++ | +++++ |
| C4 | ++ | + | ++ | ++ | +/++ | ++/+++ | ++ | + | ++ | ++ | ++/+++ | +++++ |

For each group $n = 4$ (samples labelled 1–4). Contralateral uninjected control muscles were also analysed (samples labelled C1-C4). [a]Severe damage in muscle 2. See comments in the text. *In vivo* experiments were carried out on 6–8-week-old mice. Animals were housed in a minimal disease facility with food and water *ad libitum*. In some experiments, mice were injected percutaneously into the middle of both anterior tibial muscles with 25 μl of a 1.2% solution of barium chloride in normal saline.[15] Five days later, injections of saline or plasmid DNA (1 μg/μl) in saline were administered in the same manner. All injections (25 μl) were carried out using a 27-gauge needle in a proximal-to-distal direction, under fentanyl/fluanisone and midazolam general anaesthesia (respectively, Hypnorm, Janssen Pharmaceutical, High Wycombe, UK; and Hypnovel, Roche, Welwyn Garden City, UK). All microscopical analysis was carried out on a Leica DML light microscope. Images were recorded using an Image Pro-plus package (Datacell, Maidenhead, UK) and a 3CCD colour camera (JVC). The degree of inflammation was determined by the grading of H&E and Mac-3-stained slides on a scale from + to +++++ (where + = focal inflammation with small numbers of inflammatory cells seen and +++++ = severe inflammation with several foci of infiltrating cells). Relative infiltration of $CD4^+$ and $CD8^+$ cells was determined on a – to +++++ scale where – indicates no positive cells detected, + = fewer than 10 positive cells seen and +++++ large numbers of positive cells seen, often at more than one focus of inflammation. All histological slide assessment was carried out by two independent observers. Sections for 10 different levels through the muscle were examined and the section showing the most pathology is reported.

analysis at 7 days after injection showed that there was no major difference between the immune response evoked by causing injury with a needle and that resulting from injection of normal saline. In the majority of cases, both treatments resulted in a mild mononuclear infiltrate comprised mostly of macrophages, with inflammation limited to the area surrounding the needle path and adjacent to the small centrally nucleated fibres resulting from muscle regeneration following needle damage to the fibres. Injection of plasmid DNA, however, caused a notable increase in inflammation. Again, the infiltrate was comprised mostly of macrophages, although a small increase in the number of $CD8^+$ cells was observed (Figure 1). In three out of four cases, the non-regenerated muscles injected with CMVβ had the biggest inflammatory infiltrate. Contralateral muscles which had been untreated were seen to have a small number of macrophages present, which corresponds to the level of resident macrophages in normal muscle.

The effects of intramuscular injection into regenerated muscle were more difficult to assess because of the damage already induced in the muscle by injection of barium chloride. Hence, in all groups, the level of inflammation was higher than that seen after injection into non-regenerated muscle with a proportionally larger number of $CD4^+$ and $CD8^+$ lymphocytes present. In the animals treated with needle-stick or saline injection, levels of inflammation did not seem any more severe than those seen in the contralateral muscles which had been regenerated but not subjected to any further injections. The increased inflammatory response between animals injected with pBS or CMVβ and those subjected to needle injury or saline injection was also observed in regenerated muscle, and to a much greater degree in terms of the numbers of $CD8^+$ and $CD4^+$ cells.

There are several CpG motifs present both in the ampicillin resistance gene (present in both pBluescript and CMVβ) and in the CMV immediate–early promoter sequence (on CMVβ only). Their presence probably accounts for some of the differences in inflammatory response induced by the two types of plasmid used and for the greater influx of mononuclear cells into muscles injected with plasmid compared with those treated with saline.

Another group of male C57/Bl10 mice were injected with either 25 μl normal saline or 25 μl of normal saline to which had been added 0.5 EU of LPS (equivalent to the maximum amount present in 25 μl of injected DNA used in this study). Four animals were used in each group and as before they were killed 7 days after *in vivo* treatment

Injection of bacterial LPS into non-regenerated muscle

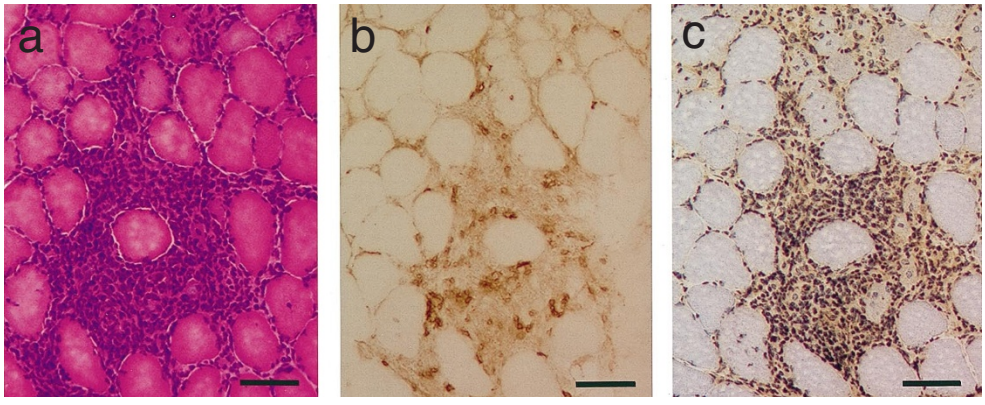


***Figure 1*** *Tibialis anterior muscle 7 days after injection with CMVβ plasmid stained with (a) H&E, (b) α-CD8 antibody and (c) α-Mac-3 antibody. Note the presence of a large inflammatory infiltrate comprised mostly of macrophages but with CD8⁺ lymphocytes also present. Centrally nucleated regenerated muscle fibres are evident indicating the muscle damage incurred at the time of injection. Scale bar indicates 25 μm. Plasmids were purified as previously described[15] with one modification: following the final clearing spin, and before addition to the column, the lysate was incubated in 1% (w/v) Triton-X114 for 30 min on ice.[46] Cleared lysate was then added to the column and washed with ×1.5 manufacturer's recommended wash buffer volume. This step reduced endotoxin levels to less than 0.02 EU per microgramme of plasmid, as assessed by the Limulus amaebocyte lysate assay (Etoxate; Sigma, Poole, UK), for the preparations used in this study. Identity was confirmed by agarose gel electrophoresis of both uncut and restriction digested plasmids. Contamination with RNA was not observed and the majority of each plasmid preparation was as covalently closed circles. At appropriate time-points after in vivo treatments, mice were killed and both tibialis anterior muscles were snap-frozen as previously described.[15] Ten-micron cryostat sections were cut at 10 evenly spaced levels throughout each muscle and lifted on to APES-coated slides.[47] Standard H&E staining was carried out to assess cell morphology and inflammatory infiltrate. X-gal staining for β-galactosidase was carried out as previously described.[15] Sections for immunohistochemistry were stained using antibodies to CD4 (YTS191) at 1:2 dilution and CD8 (YTS169) at 1:2 (a gift from David Baker, Institute of Ophthalmology) and Mac-3 (M3/84 Pharmingen, San Diego, CA) at 1:50 using standard techniques. Mac-3 slides were counter-stained in haematoxylin.*

produced a larger inflammatory infiltrate and a greater number of regenerated fibres than was seen after injection of saline alone. However, this was less pronounced than the inflammatory response following plasmid injection. The infiltrating cells were predominantly macrophages with small numbers of $CD4^+$ and $CD8^+$ cells present (data not shown).

In a further experiment C57/Bl10 mice were injected intraperitoneally (i.p.) with (a) normal saline, (b) rat IgG, (c) rat IgG α-CD4 antibodies or (d) rat IgG α-CD8 antibodies at days –3, 0 and 3 relative to intramuscular injection with (1) normal saline, (2) pBS or (3) CMVβ. Animals were injected in both tibialis anterior muscles and were killed 7 days after intramuscular injection.

Results are reported in Table 2. Histological analysis of haematoxylin and eosin (H&E)-stained muscle sections from all groups demonstrated that pathological changes due to needle damage and infiltration of mononuclear cells were not dependent on intraperitoneal treatment. Depleting antibodies diminished, or in most cases ablated, the number of T lymphocytes present in the inflammatory infiltrate. However, the salient histological features of the muscles from animals treated with depleting antibodies were essentially the same as the muscles from the two control groups of animals. In all groups, the greatest inflammation was seen when plasmid DNA, both plasmid backbone or plasmid expressing a neo-antigen (β-galactosidase), was injected intramuscularly. Intraperitoneal administration of rat monoclonal antibodies did not evoke an immune response in the mouse muscle, since no difference was observed between animals injected i.p. with saline and those injected i.p. with rat IgG.

Transfection analysis in 3T3 fibroblasts showed that methylation of CMVβ (mCMVβ) reduced the expression levels 67-fold compared with non-methylated CMVβ. Digestion of plasmid with *Psp1406* before transfection reduced expression from unmethylated DNA but there was only a slight reduction in expression from digested methylated DNA indicating that some expression was being driven from hemimethylated or methylated CMV promoter sequences and that the plasmid was highly methylated (data not shown). To assess the *in vivo* titration of expression from methylated plasmid, tibialis anterior muscles were injected with either 25 μg of methylated CMVβ or one of the following mixtures of unmethylated CMVβ plus pBluescript (pBS) to maintain an equal quantity of DNA: 25 μg CMVβ + 0 μg pBS, 2.5 μg CMVβ + 22.5 μg pBS or 0.25 μg CMVβ + 24.75 μg pBS. Four muscles were injected for each condition. Each mixture also contained 2.5 μg of RSVCAT plasmid. The muscles were harvested at 1 week and analysed for CAT and β-galactosidase expression. After correction for the cotransfection control, the level of CMVβ plasmid that gave the same expression *in vivo* as 25 μg of methylated CMVβ was calculated from the standard curve of expression *versus* quantity of plasmid injected for CMVβ. The relative reduction in expression *in vivo* following methylation was approximately 42-fold. This compares well with the differences in expression levels seen in the *in vitro* expression study. From the *in vivo* titration experiment, it was determined that 0.6 μg of CMVβ gave the same level of β-galactosidase expression as 25 μg of mCMVβ.

The reduction in overall inflammation following injection of methylated plasmid was dramatic. Histological analysis of muscles taken at 3 and 14 days after injection (summarised in Table 3) revealed that muscles injected with methylated plasmid had generally lower levels of inflammatory infiltrate, as well as fewer $CD4^+$ and $CD8^+$ cells, than muscles injected with CMVβ. Figure 2 shows histology of example sections. The thymus weights rela-

**Table 2** Histological analysis of muscles

| *I.V. Treatment* | *Injection* | *Inflammation* | *CD4* | *CD8* |
|---|---|---|---|---|
| *(a) Muscles treated with depleting antibodies* | | | | |
| a-CD4 Ab | Saline | + | − | + |
| a-CD4 Ab | Saline | + | − | + |
| a-CD4 Ab | Saline | ++ | − | +/++ |
| a-CD4 Ab | Saline | ++ | − | + |
| a-CD4 Ab | pBS | ++ | − | ++ |
| a-CD4 Ab | pBS | +++ | + | ++/+++ |
| a-CD4 Ab | pBS | ++/+++ | +/− | ++/+++ |
| a-CD4 Ab | pBS | ++ | − | +/++ |
| a-CD4 Ab | CMVβ | ++++ | ++ | ++ |
| a-CD4 Ab | CMVβ | ++/+++ | + | +++/++++ |
| a-CD4 Ab | CMVβ | ++/+++ | +/− | ++/+++ |
| a-CD4 Ab | CMVβ | +++ | + | ++/+++ |
| a-CD8 Ab | Saline | ++ | − | − |
| a-CD8 Ab | Saline | +/++ | − | − |
| a-CD8 Ab | Saline | +/++ | − | − |
| a-CD8 Ab | Saline | +/++ | − | − |
| a-CD8 Ab | pBS | ++ | ++ | − |
| a-CD8 Ab | pBS | ++ | +/++ | − |
| a-CD8 Ab | pBS | ++ | + | − |
| a-CD8 A8 | CMVβ | ++ | ++/+++ | +/++ |
| a-CD8 A8 | CMVβ | +/++ | ++ | +/++ |
| a-CD8 A8 | CMVβ | ++/+++ | +/++ | + |
| a-CD8 A8 | CMVβ | ++/+++ | +/++ | +/++ |
| *(b) Control muscles (i.v. treatment with normal saline or rat IgG)* | | | | |
| Normal saline | Saline | + | − | + |
| Normal saline | Saline | + | − | + |
| Normal saline | Saline | ++ | − | + |
| Normal saline | Saline | ++ | − | + |
| Normal saline | pBS | + | − | +/++ |
| Normal saline | pBS | + | +/++ | + |
| Normal saline | pBS | +/++ | + | ++ |
| Normal saline | pBS | ++++ | ++ | +++/++++ |
| Normal saline | CMVβ | +++ | + | +++ |
| Normal saline | CMVβ | ++/+++ | ++ | ++ |
| Normal saline | CMVβ | ++/++++ | ++ | ++/+++ |
| Normal saline | CMVβ | + | + | +/++ |
| IgG | Saline | + | − | + |
| IgG | Saline | + | − | + |
| IgG | Saline | +/++ | +/++ | + |
| IgG | Saline | + | + | − |
| IgG | pBS | +/++ | + | +/++ |
| IgG | pBS | +/++ | + | ++ |
| IgG | pBS | +/++ | +/++ | +/++ |
| IgG | pBS | ++/+++ | ++ | + |
| IgG | CMVβ | ++ | + | ++ |
| IgG | CMVβ | ++/+++ | + | ++ |
| IgG | CMVβ | ++ | ++/+++ | ++ |

Groups of animals were injected intraperitoneally (i.p.) with (a) normal saline; (b) 500 μg rat IgG; (c) 500 μg rat IgG α-CD4 antibodies; or (d) 500 μg rat IgG α-CD8 antibodies at days −3, 0 and 3 relative to intramuscular injection with (1) 25 μl normal saline; (2) 25 μl pBS; or (3) 25 μl CMVβ. The depleting antibody mixes were purified immunoglobulin at 10 mg/ml and consisted of the following antibodies: α-CD4, YTS191 plus YTA3; α-CD8, YTS169 plus YTS156. These antibodies were a kind gift from Steve Cobbold, Oxford. Animals were injected in both tibialis anterior muscles and were killed 7 days after intramuscular injection.

tive to body weight were significantly lower at the 3-day time-point in the animals treated with methylated compared with unmethylated CMVβ ($P < 0.05$) indicative of an overall reduction in proliferative immune responses. Although the quantity of plasmid injected and the initial expression level of β-galactosidase was matched between the two groups, the antibody response to β-galactosidase at 14 days was significantly reduced ($P < 0.033$, Student's *t* test) in the group treated with methylated plasmid (Figure 3). There was no detectable IgG response at 3 days. β-Galactosidase expression was significantly increased ($P < 0.05$) at the 14 day time-point in the group treated with methylated plasmid (Figure 4). In our earlier studies, all β-galactosidase expressing fibres were eliminated by 2 weeks by Th1-mediated CTL response (Maule *et al*, manuscript in preparation). The data from the methylation study would suggest that prolonged expression of even the highly immunogenic prokaryotic

**Table 3** Immunohistochemical analysis of muscles injected with CMVβ and mCMVβ at 3 and 14 days after injection

| | *Time-point (days)* | *Muscle* | *Inflammation (as assessed by H&E)* | *CD4* | *CD8* | *Mac-3* |
|---|---|---|---|---|---|---|
| CMVβ | 3 | 1 | +++ | ++ | +++ | ++++ |
| | | 2 | +++ | ++ | ++ | +++ |
| | | 3 | ++++ | ++/+++ | +++ | ++++ |
| | | 4 | ++++ | ++/+++ | ++/+++ | ++++ |
| *mCMVβ | 3 | 1 | +++++ | + | + | ++++ |
| | | 2 | + | + | + | +++/++++ |
| | | 3 | + | +/− | + | +++/++++ |
| | | 4 | + | +/++ | +/++ | +++ |
| CMVβ | 14 | 1 | +++ | +++/++++ | ++++/+++++ | +/− |
| | | 2 | ++/+++ | +++/++++ | ++++ | +/++ |
| | | 3 | +++ | +++/++++ | ++++ | + |
| | | 4 | ++/+++ | ++++ | ++++ | ++ |
| mCMVβ | 14 | 1 | − | +/− | − | − |
| | | 2 | ++ | +++ | ++/+++ | + |
| | | 3 | +++ | +++ | +++ | + |
| | | 4 | + | − | − | +/− |

*Severe damage in muscle 1. See comments in the text.

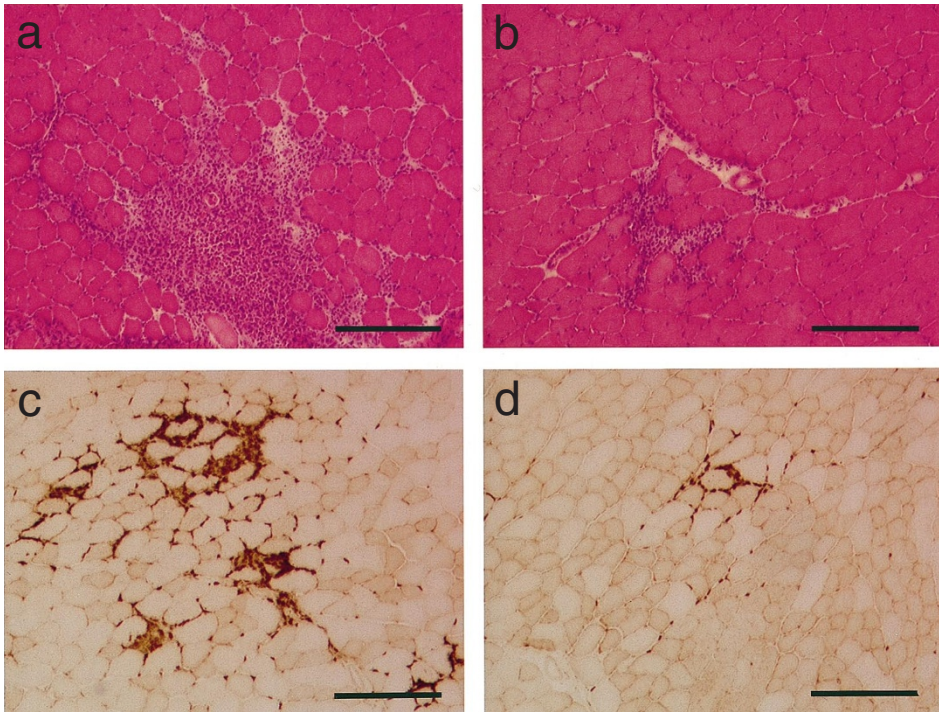


***Figure 2*** *Photomicrographs of transverse sections from muscle injected with (a) unmethylated CMVβ and (b) methylated CMVβ sampled 3 days after injection and stained with H&E. Methylation of the plasmid results in a smaller lesion and fewer regenerated fibres. (c and d) Sections stained with α-CD8 antibody from muscles injected 14 days previously with (c) unmethylated CMVβ and (d) methylated CMVβ. Again, injection of methylated plasmid is seen to result in a smaller inflammatory lesion and a reduced infiltrate of $CD8^+$ lymphocytes. Scale bar indicates 100 μm. Plasmid DNA was methylated using Sss1 methylase (CpG methylase; New England Biolabs, Hitchin, UK) according to the manufacturer's instructions. Methylation was more than 90% complete as assessed by restriction digests with the methylation sensitive restriction enzymes Not1 and Psp1406 and gel electrophoresis. Alu1 restriction was not altered by methylation (data not shown).*

β-galactosidase antigen could be obtained in immunocompetent mice following direct injection of plasmid DNA. Klinman *et al*[34] have shown that methylation with *Sss*I methylase of a plasmid used for vaccination reduced the immunogenicity of the vaccine when assayed after a boost injection. Addition of excess unmethylated DNA to the methylated or unmethylated vaccination plasmid boosted the immune response *in vivo* demonstrating the strong immunostimulatory capacity of the unmethylated CpG motifs. The degree of methylation was inversely proportional to the ability of the plasmid to induce cytokine responses *in vitro*. Our analysis of the inflammation at early time-points after injection is in agreement with their conclusions.

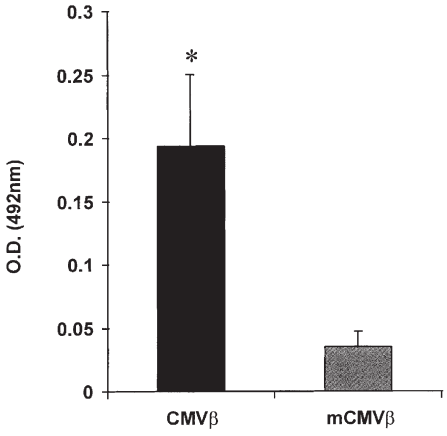


***Figure 3*** *β-Galactosidase-specific IgG response at 14 days following injection of unmethylated (CMVβ) or methylated (mCMVβ) plasmid. Error bars indicate s.e.m. *P = 0.0333. β-Galactosidase antibody ELISAs were carried out as previously described*[15] *except that bound antibody was detected with rabbit anti-mouse pan-IgG horseradish peroxidase used at 1:4000 (BioRad, Hemel Hempstead, UK). The background reading from wells containing all reagents except the coating antigen was subtracted for each data-point.*

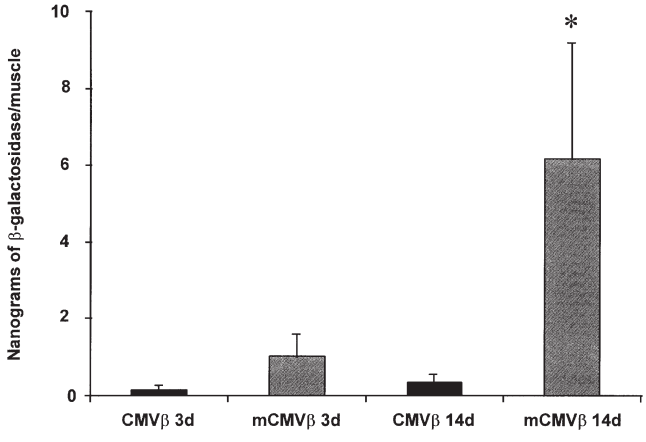


***Figure 4*** *Total expression of β-galactosidase in tibialis anterior muscles injected with unmethylated (CMVβ) or methylated (mCMVβ) plasmid and assayed at 3 or 14 days after injection. Error bars indicate s.e.m. *P < 0.05 for mCMVβ at 14 days compared with all other data sets. Two groups of eight male 6–8-week-old F1 mice, from C57/Bl10 males crossed with CBA/J females (Harlan Olac, Bicester, UK), were injected with either 20 μg of methylated CMVβ plasmid (mCMVβ) or 0.5 μg CMVβ (with 19.5 μg pBS added). At 3 and 14 days after injection, four of each group of animals were killed and blood samples were collected by cardiac puncture under terminal anaesthesia. One of each pair of tibialis anterior muscles was analysed for total β-galactosidase expression using the Boehringer (Lewes, UK) β-galactosidase ELISA kit as previously described.*[15]

Two incidences are marked on Tables 1a and 3 in which a single animal in a group of four showed an extreme level of inflammation following simple needlestick or injection of plasmid, respectively. These muscles showed large areas of muscle degeneration and regeneration, as revealed by centrally nucleated fibres, which is in marked contrast to the limited areas of central nucleation seen in the other muscles. We hypothesise that in these cases the path of the needle had caused occlusion of a blood vessel or caused local nerve damage such that a much larger number of fibres were involved. The lower number of damaged fibres in the other muscles reflects direct damage to fibres along the needle path. Stochastic events of this nature may be part of the explanation for the considerable variation seen in this technique.[35,36]

The exact mechanisms by which plasmid DNA can exacerbate inflammatory lesions have not yet been fully elucidated. Contaminating bacterial LPS in the plasmid preparations can act as an adjuvant. It is known to be a ligand for the macrophage scavenger receptor[24] and has been implicated in the up-regulation of cytokine production by a variety of cell types *in vitro*.[23] However, its effect on muscle pathology when injected alone is not as great as that produced by plasmid injection, implying that, in this case, it is likely to act synergistically with other factors.

In previous studies, prolongation of transgene expression following administration with viral vectors has been achieved by ablation of T cell subsets with monoclonal antibodies to CD4 and CD8.[37–40] However, the contribution of these T cell subsets to the initial inflammatory lesions has not previously been examined. The apparent failure to achieve complete T cell ablation in our current study is probably the consequence of retention of an intact thymus coupled with a less than 100% ablation. The lack of sensitivity of the inflammatory lesion to the presence of T cells, along with immunocytochemistry for Mac-3, suggests that the major components of the inflammatory infiltrate are monocyte-derived macrophages. Although such cells are not directly involved in the Th1 pathways responsible for loss of fibres expressing transgenes, their induction of inflammatory cytokines will produce an environment within the injected muscle more likely to recruit immune cells into the tissue or the draining lymph node. This will speed up the process by which fibres expressing transgenes are eliminated. A second and potentially more damaging effect of macrophages in the muscle is their ability to present foreign antigen by means of an MHC class1 pathway.[41] The proposed 'crossover' pathway for antigen presentation implies that bone marrow-derived antigen-presenting cells, such as macrophages, can act as mediators of antigen-specific immune reactions.

Methylation of the plasmid DNA dramatically reduced expression of the β-galactosidase reporter gene both *in vitro* and *in vivo* consistent with results previously described.[42] However, two problems are associated with methylation of plasmid DNA. It was noticeable that our larger scale preparations of methylated DNA were less readily solubilised for injection. The insolubility is possibly due to a greater tendency of methylated DNA to form zDNA.[43,44] Additionally, methylation can down-regulate promoter activity although there are examples of promoter sequences devoid of CpG motifs, ie mouse mammary tumour virus long terminal repeat (MMTV LTR), which renders such promoters insensitive to methylation.[45] Unfortunately, the expression levels obtained from MMTVβ following direct injection are lower than that obtained for methylated CMVβ (Wells KE, unpublished data).

If plasmids are to be used successfully for the purposes of local gene therapy, or for a means of providing a systemic source of therapeutic proteins, great care must be taken when designing constructs. The use of this technology will require one or more of the following modifications: (1) bulk methylation of CpG motifs; (2) the use of strong methylation insensitive promoters; (3) the use

of antibiotic resistance genes and promoter elements containing fewer immunostimulatory sequences; and (4) the use of plasmid purification procedures designed to further reduce contaminating LPS. Without such developments, the use of plasmid DNA in clinical gene therapy is likely to be limited due to the substantial muscle damage incurred.

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