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REGULAR ARTICLE

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Silencing TNFα activity by using Remicade or Enbrel blocks inflammation in whole muscle grafts: an in vivo bioassay to assess the efficacy of anti-cytokine drugs in mice

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Abstract Dramatic clinical success in the treatment of chronic inflammatory diseases has resulted from the use of anti-cytokine therapies including specific blocking antibodies, soluble receptors and traps to silence the actions of inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and interleukin-1 (IL-1). Two agents used clinically to block the functional activity of TNFα protein are Remicade (an antibody) and Enbrel (a soluble TNF receptor). These tools are now being extended to many other clinical disorders. We have a specific interest in the treatment of muscle diseases. In order to study the effects of novel anti-cytokine drugs on mouse models of human disease, such drugs must be investigated to determine whether they are indeed effective in blocking the inflammatory response in mouse. This has been carried out by means of a simple in vivo bioassay. Histological examination of transverse sections from whole muscle autografts in C57BL/10ScSn mice sampled at 5 days after transplantation provides an excellent assay model and clearly shows that Remicade and Enbrel block the acute inflammatory cell response in vivo. This graft model has also been used to show that a single intraperitoneal injection of Remicade (10 μg/g) is long-lived and effective when administered at 1 week and even 4 weeks prior to the assay. Enbrel is highly effective when injected twice at −3 days and −1 day (2×100 μg) before muscle grafting but shows no inhibition of the inflammatory response after a single injection (100 μg) 1 week prior to grafting. This striking ablation of inflammation by pharmacological blockage of TNFα is in marked contrast to the lack of any effect in TNFα null mice. This simple reproducible in vivo assay model in mice can be used to evaluate the efficacy of many novel anti-cytokine interventions designed to block inflammation.

Keywords Remicade · Enbrel · TNFα · Inflammation · Muscle · Mouse (female C57BL/10SnSc)

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Introduction

The pharmacological blockade of inflammatory cytokines is an increasingly attractive clinical therapy for inflammatory disorders, such as rheumatoid arthritis (Graninger and Smolen 2002; Goldbach-Mansky and Lipsky 2003) and Crohn's disease (Ogata and Hibi 2003; Korzenik 2004); its use has also been considered in many other situations, including central nervous system ischaemia (Clark and Lutsep 2001), heart failure (Anker and von Haehling 2004; Khanna et al. 2004; Wolfe and Michaud 2004) and infections (Dinarello 2003). Tumour necrosis factor alpha (TNFα) is a key cytokine that stimulates the inflammatory cell response (Arend 2002). Two agents used clinically to block the functional activity of TNFα protein are a chimeric monoclonal antibody called Remicade (also known as Infliximab), which is composed of murine variable and human constant regions (Graninger and Smolen 2002; Scallon et al. 2002), and a soluble TNF receptor (TNFR) called Enbrel (Etanercept), which is a dimeric fusion protein composed of an extracellular ligand-binding portion of the human (p75) TNFR linked to the Fc portion of human IgG1 (Feldmann and Maini 2001; Mikuls and Moreland 2001). Enbrel inhibits the binding of both TNFα and TNFβ (lymphotoxin alpha) to cell surface TNFRs, rendering TNF biologically inactive. We are interested in such anti-cytokine therapy to treat muscle diseases. For experimental purposes, it is highly desirable to test whether such clinically relevant drugs also prevent inflammation in mouse models.

We have recently shown that Remicade greatly reduces the extent of acute skeletal muscle necrosis and the dystropathology in the mdx mouse model for the lethal childhood

muscle disease, Duchenne muscular dystrophy (DMD); (Grounds and Torrisi 2004), thereby revealing a novel role for such cytokines in the breakdown of skeletal muscle. This presents the opportunity for immediate clinical intervention by using anti-cytokine therapies to treat muscular dystrophies. In order to establish that a range of anti-cytokine drugs used in clinical studies are indeed effective at blocking the inflammatory response in mouse models of human disease, a simple in vivo bioassay is required. Here, we describe the use of whole muscle autografts in mice (Roberts and McGeachie 1992; White et al. 2000) to demonstrate that both Remicade and Enbrel impair the inflammatory cell response: inflammation is normally one of the key early events that precedes new muscle formation and regeneration (Robertson et al. 1993). This simple model allows drug doses and administration regimes to be optimised with special reference to the time that a single injection of the drug is effective and can easily test the efficacy of new batches of drugs before experimental use in mice.

Materials and methods

Animals

All experiments were carried out on female C57BL/10SnSc mice (the background normal strain for mdx mice) aged between 4–6 weeks; the mice were specific pathogen-free and obtained from the Animal Resources Centre Murdoch, Western Australia. Mice were housed and treated according to the Western Australian Prevention of Cruelties to Animals Act (1920), the National Health and Medical Research Council and the University of Western Australia Animal Ethics Committee.

Drug administration

Remicade (Schering-Plough, Australia) was injected intraperitoneally at a dose of 10 μg/g body weight (250 μg in a final volume of 100 μl, for a 25 g mouse). Control mice were injected with an equivalent amount of the carrier molecule, mouse serum albumin (MSA), that was used for Remicade. Mice were injected with Remicade either at 2, 7, 14 or 28 days prior to whole muscle grafting in regimes as summarised in Tables 1, 2, and 3.

Enbrel (Wyeth, Australia) was injected as two doses of 100, 200 and 500 μg each (for average body weight of 25 g) intraperitoneally in a pilot study at −3 days and −1 day before transplantation. As a result of this pilot study, the 100 μg dose for each injection was used for subsequent whole muscle graft experiments. Control mice were injected with an equivalent dose of non-specific human IgG (Sigrma), which is the appropriate control for Enbrel. Treated mice were injected with Enbrel either at −3 and −1 days or at −7 days only, prior to whole muscle grafting in regimes as summarised in Tables 1, 2, and 3.

Whole muscle graft surgery and sampling

Mice were anaesthetised with 1.5% (v/v) Rodia Halothane (Merial), N_2O and O_2 and autografts of whole intact extensor digitorum longus (EDL) muscles were performed on both legs as described in detail elsewhere (Roberts and McGeachie 1992; White et al. 2000). Briefly, the EDL muscles with both tendons attached were removed from the anatomical bed and transplanted onto the surface of the tibialis anterior (TA) muscle. The tendons were sutured onto the underlying tissues. The skin was closed and the autografts were allowed to regenerate.

It is well documented that most of the myofibres in the grafted muscle (in which all blood and nerve supplies have been severed) undergo necrosis, although some myofibres survive at the periphery. New muscle formation to replace the necrotic tissue starts at the edge of the graft in response to the infiltration of inflammatory cells that are closely associated with revascularisation. By 5 days after transplantation, the inflammatory zone is normally well advanced. Much of the necrotic tissue has been phagocytosed and myoblasts are activated, proliferate and fused to form

Table 1 Extent of persisting necrotic muscle tissue, which inversely reflects the activity of inflammatory cells, and of new muscle formation in whole muscle autografts of C57BL/10ScSn mice sampled at 5 days after transplantation. The average area of remaining necrotic muscle tissue (as a percentage of the total graft area) ± standard deviation (shown in brackets) is presented for grafts from control (untreated, MSA-injected and HIgG-injected), Remicade- and Enbrel-treated mice. The area of persisting necrotic tissue was significantly larger in grafts from Enbrel-treated (−3 days and −1 day) mice compared with all control groups. Since there were only two Remicade (−2 days) grafts available for analysis, no statistical analysis was performed for this group. The density of myotubes within the regenerated zone (and standard deviation) is also shown. Since inflammatory cells are required for the removal of necrotic tissue and new muscle formation, the extent of regeneration directly reflects the strength of the inflammatory cell response

Treatment prior to grafting	% Persisting necrotic tissue	Myotube density within regenerating zone	Comment on extent of regeneration
Untreated control (n=4)	48.70 (10.0)	0.87 (0.44)	Advanced but incomplete
MSA control (n=4)	25.39 (12.9)	4.23 (3.95)	Advanced but incomplete
HIgG control (n=4)	36.70 (13.7)	1.42 (1.32)	Advanced but incomplete
Remicade (−2 days) (n=2)	64.22 (21.22)	1.61 (0.82)	Strongly inhibited
Enbrel (−3 days and −1 day) (n=6)	77.40 (10.1)*	1.28 (0.53)	More strongly inhibited

*Statistical significance (P<0.005)

Table 2 Effect of anti-TNFα drugs administered at various times prior to grafting on whole muscle autografts of C57BL/10ScSn mice sampled at 5 days after transplantation. The average area of remaining necrotic muscle tissue (with standard deviation) is shown for grafts from Remicade- and Enbrel-treated mice (other details as for Table 1)

Treatment prior to grafting	% Persisting necrotic tissue	Myotube density within regenerating zone	Comment on extent of regeneration
Remicade (−7 days) (*n*=6)	51.31 (23.6)	3.32 (0.98)	Strongly inhibited
Remicade (−14 days) (*n*=4)	75.42 (9.9)	5.47 (5.32)	Inhibited
Remicade (−28 days) (*n*=6)	59.66 (3.78)	2.72 (2.34)	Inhibited
Enbrel (−7 days) (*n*=4)	28.10 (26.0)	0.1 (0.05)	Similar to controls (see Table 1)

myotubes (multinucleated young muscle fibres), which are conspicuous in the regenerating peripheral zone. The centripetal pattern of regeneration is similar throughout the length of the graft (unpublished data): this conveniently means that all transverse section show a similar histological pattern.

All grafts were routinely sampled at 5 days after transplantation for comparative morphometric analysis (Tables 1, 2); four grafts from duplicate mice (*n*=4) were analysed unless otherwise indicated. However, some grafts were sampled at earlier (3 days) or later (7 days or 14 days) time points (see Table 3). Mice were killed by cervical dislocation. The entire TA muscle with the overlying EDL graft was carefully dissected, immediately fixed in 4% (w/v) paraformaldehyde (pH 7.6) for 30 min, transferred to 70% (v/v) ethanol and processed in a Shandon automatic tissue processor. Muscle grafts were dissected in the mid-region and embedded with both cut surfaces at the top of the paraffin block. Transverse sections (5 μm) were collected on glass slides and stained with haematoxylin and eosin (H&E) for histological examination and morphometric analysis.

Morphometric analysis

The area within the graft of persisting necrotic muscle tissue was quantitated by detailed morphometric analysis on sections stained with H&E and was expressed as a proportion of the total transverse graft area (necrotic tissue persists in the absence of inflammatory cells). The remainder of the transverse section of the graft was occupied by the regenerating zone that consisted of an inflammatory cell infiltrate and new muscle cells (both myoblasts and myotubes). Slides were placed on a Leica PM RBE microscope that was connected to a personal computer and attached to a video camera (Hitachi HV-C20M). Non-overlapping images of the entire grafts were taken and tiled together by using ImagePro Plus 4.0 (Media Cybernetics) software and an automated microscope stage movement mechanism. The total area of each graft and the area of inflammatory cell infiltration/regeneration within each graft was measured by using ImagePro. The area of persisting necrotic tissue was determined by subtracting the value (%) of inflammatory cell infiltration/regeneration from the entire area of the graft (100%). In addition, the density of myotubes in the regenerating zone was measured to determine whether the investigated drugs impaired the ability to form new muscle (myotubes appeared in transverse sections as plump basophilic cells with at least one centrally located nucleus). A rim of surviving original myofibres seen at the edge of many

Table 3 Response of whole muscle autografts sampled at 3, 7 or 14 days after transplantation, for a range of treatment regimes. The area of persisting necrotic tissue was significantly larger in day-7 Remicade-treated (−2 days) grafts compared with day-7 MSA control grafts (other details as for Table 1)

Sampling (days)	Treatment prior to grafting	% Persisting necrotic tissue	Myotube density within regenerating zone	Comment on extent of regeneration
3	Untreated (*n*=3)	63.21 (9.85)	0.74 (0.33)	Minimal
3	Remicade (−2 days) (*n*=3)	88.19 (7.79)	(no myotubes)	Minimal
3	Enbrel (−3 and −1 day) (*n*=2)	92.49 (6.10)	0.37 (0.07)	Minimal
3	Enbrel (−7 days) (*n*=2)	94.67 (1.24)	(no myotubes)	Minimal
7	Untreated (*n*=2)	8.69 (9.50)	9.80 (3.61)	Well advanced
7	MSA control (*n*=6)	5.6 (2.31)	10.44 (2.81)	Well advanced
7	HIgG control (*n*=4)	14.56 (6.4)	3.94 (3.47)	Well advanced
7	Remicade (−2 days) (*n*=6)	16.48 (6.56)*	18.22 (1.54)	Well advanced
7	Enbrel (−3 days and −1 day) (*n*=4)	7.88 (7.65)	21.1 (7.6)	Well advanced
7	Enbrel (−7 days) (*n*=2)	7.50 (2.09)	17.5 (0.4)	Well advanced
14	MSA control (*n*=5)	2.43 (1.24)	(all myotubes)	Regeneration complete
14	Remicade (−2 days) (*n*=6)	9.43 (14.17)	(all myotubes)	Regeneration complete

*Statistical significance ($P<0.005$)

grafts occupied less that 5% of the total graft area and was simply excluded from the analysis.

Results

Comparison of whole muscle grafts sampled at day 5

The areas of persisting necrotic tissue are summarised in Table 1. This measurement was used as it inversely reflected the activity of inflammatory cells, i.e. a larger area of persisting necrotic tissue indicated the absence of inflammatory cells. The overall appearance of autografts at 5 days after transplantation was similar for all three control groups (untreated, MSA- or HIgG-injected C57BL/10 SnSc mice); a typical histological appearance is shown in Fig. 1a. At day 5 in control grafts, much of the necrotic tissue had been removed as a result of phagocytosis by inflammatory cells that had entered the graft (Fig. 1a). The removal of necrotic tissue by inflammatory cells was associated with new muscle formation and a well-advanced zone of regeneration, which contained inflammatory cells and myotubes occupying much of the tissue section and extending from the periphery towards the centre of the graft, where some necrotic tissue still remained (Fig. 1a). Sometimes, the zone of persisting necrotic tissue was more pronounced, as illustrated for another control graft in Fig. 1b. Biological variation was noted between animals and even between autografts in both legs of an individual mouse (reflected in the standard deviations), although the overall trend was consistent within each group, as indicated in Table 1. The more mature myotubes, which had formed

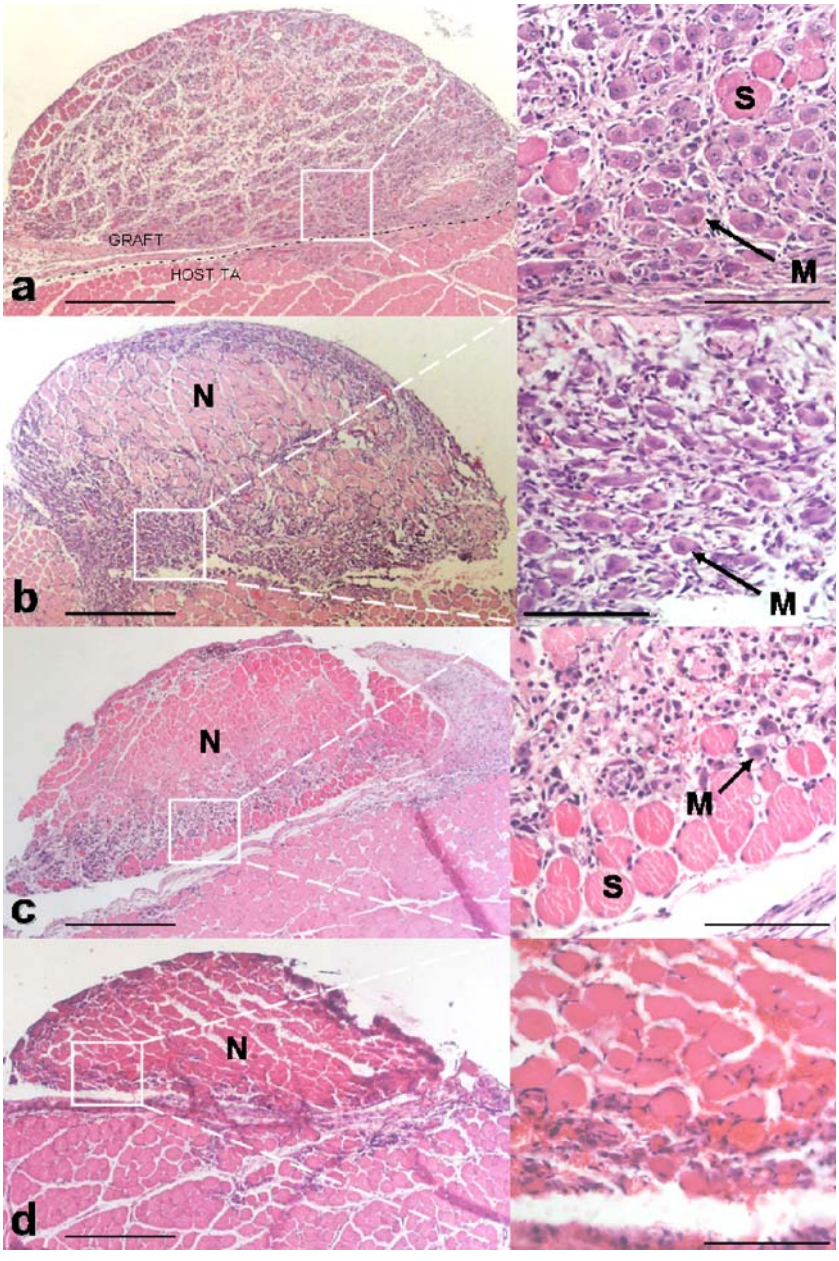

Fig. 1 Pattern of regeneration in whole muscle autografts in C57BL/10ScSn mice sampled at 5 days after transplantation. Control mice injected prior to grafting with (**a**) mouse serum albumen (MSA) or (**b**) non-specific Human IgG are compared with mice injected with (**c**) Remicade and (**d**) Enbrel (2×100 μg dose). Low-power images (*left*) show a transverse section through the mid-region of a whole muscle autograft overlying the TA muscle; a central zone of persisting necrotic tissue (*N*) is conspicuous in **b**–**d**. The high-power view (*right*) of an area at the periphery of the same graft shows some surviving myofibres (*S*) that lack central nuclei and many plump myotubes with central nuclei (*M*). *Bars* 400 μm (*left*), 100 μm (*right*)

first, were seen at the periphery of the graft (see high-power views in Fig. 1a, b), although their density varied throughout this regenerating zone. Control untreated grafts in C57BL/10 SnSc mice at 5 days were similar to MSA-injected (Fig. 1a) and HIgG-injected (Fig. 1b) controls (see Table 1).

In mice injected with Remicade at 2 days prior to grafting, the appearance of grafts sampled at 5 days was different (Fig. 1c) from controls (Fig. 1a, b), as persistant necrotic tissue occupied more (64%) of the graft area. Few inflammatory cells were present and myotubes were rare in the small zone of regenerative activity at the periphery of the grafts.

The appearance of grafts sampled from mice injected twice with 100 μg Enbrel (Fig. 1d) at 3 days and 1 day prior to grafting was strikingly similar to grafts from Remicade-treated mice, although the inhibition of the inflammatory response was more pronounced than with Remicade (compare Fig. 1c and d). Comparison of three doses of Enbrel (100, 200 and 500 μg) showed identical results for 100 μg and 200 μg. However, the higher dose of 500 μg appeared to have little effect and the appearance of the two grafts resembled the controls, with many (95%) inflammatory cells and virtually no necrotic tissue remaining at 5 days (data not shown). Because of this, an individual dose of 100 μg Enbrel was chosen for all experiments.

Single dose effectiveness of TNFα-blocking agents

Determination of the length of time that a single dose of Remicade or Enbrel was effective at blocking inflammation was investigated by using mice injected at various intervals/times prior to grafting. The results obtained for 5-day grafts are summarised in Table 2. A single dose of Remicade at −7 days showed a similar response compared with mice given a single dose at 2 days prior to grafting (compare Tables 1, 2), with persisting necrotic tissue occupying much (51%) of the graft area. When Remicade was injected 14 days before grafting, the day-5 muscle grafts had large amounts of persisting necrotic tissue (about 75%). However, when Remicade was injected at −28 days before grafting, the results were similar to the other times of administration (−2 and −7 days) with persisting necrotic tissue occupying about 60% of the graft area. These results showed that a single dose of Remicade given up to 4 weeks before grafting still inhibited inflammation to some extent in r5-day grafts and indicated the long-lasting effect of this blocking antibody.

In contrast, a single dose of Enbrel given at 7 days prior to grafting resulted in no difference in the pattern of regeneration in grafts between control and treated mice (see Table 2). This contrasted with the strong inhibitory effect observed following two injections at 3 days and 1 day before grafting (see Table 1) and indicated that Enbrel had a much shorter effective half-life in vivo compared with Remicade and that it requires more frequent administration to block TNFα protein function.

Comparison of grafts from mice sampled at 3, 7 and 14 days after transplantation

The effects of the TNFα-blocking agents on the earlier pattern (inflammatory cell infiltration associated with revascularisation) and longer-term pattern (myotube formation and maturation associated with complete removal of all necrotic muscle tissue) of graft regeneration were studied in muscles sampled at 3, 7 and 14 days after grafting (Table 3). In untreated control mice sampled at 3 days, a large area of necrotic tissue remained (about 63%), with the rest of the tissue containing leukocytes and rarely (<1%) myotubes. In all treated mice sampled at 3 days, there was virtually no difference in the pattern of regeneration compared with controls, except for a slightly higher (about 88–95%) area of remaining necrotic tissue and almost no infiltrating leukocytes or new myotubes (Table 3).

By day 7, little necrotic tissue remained (5%–15%) and most of the tissue had regenerated in control grafts (Table 3). Myotubes were larger by this time and accordingly occupied a greater part of the regenerated zone. A similar pattern was seen for mice treated with Remicade or Enbrel, regardless of the timing of the drug administration. By 14 days after transplantation, again no difference was seen between Remicade-injected and control grafts; regeneration was essentially complete and large mature myotubes occupied 100% of the area of the regenerated tissue. These results showed that these anti-cytokine drugs did not prevent the overall process of muscle regeneration, an aspect clearly important for potential clinical application to myopathies. The inhibition of inflammation seen in 5-day grafts was not found at later times (7 and 14 days) emphasising the crucial timing for graft examination during the early stages of the inflammatory response (day 5).

Discussion

The employed model of whole muscle autograft regeneration (Roberts and McGeachie 1992; White et al. 2000) has clearly demonstrated that both Remicade and Enbrel impair the inflammatory cell response in mice. Simple regimes of injection of these pharmacological agents designed to block TNFα function result in reduced leukocyte infiltration and accordingly delayed muscle regeneration at 5 days after transplantation. Remicade has the longest lasting affect, with a single dose being partially effective at reducing inflammation (and delaying regeneration) for up to 4 weeks in grafts. However, Enbrel is not effective when administered 1 week before transplantation, indicating that the effective half-life of this drug in mice is a matter of days; this parallels the period of effectiveness (half-life of approximately 4–5 days) in clinical use when Enbrel at 25 mg is administered twice weekly in juveniles (Immunex data sheet 10662-12). Enbrel appears to be more effective than Remicade in reducing the inflammatory response if given close to the time of transplantation. This correlates with another study comparing the use of Remicade and Enbrel for in vitro binding and cell-based assays (Scallon et al.

2002). These differences might have significant implications for clinical applications.

In our experimental grafts, TNFα increases as a result of the transplantation trauma and associated muscle necrosis and regeneration (Collins and Grounds 2001; Grounds and Torrisi 2004) but there is no repeated stimulus to maintain the elevated TNFα. The immune response to the acute situation of a sustained large mass of necrotic tissue (as in the muscle graft) is markedly different from more subtle situations such as those that occur in many myopathies (and exercise-induced damage) in which a small transitory membrane lesion may result in a local inflammatory response that directly contributes to additional damage, thereby exacerbating myofibre necrosis (Grounds and Torrisi 2004). This should be considered when extrapolating our data to other in vivo situations. The short-term acute increase in TNFα and the inflammatory response in grafted or injured muscles contrasts with, for example, the repeated muscle breakdown that occurs in muscular dystrophies and the relatively low TNFα in mdx mice (discussed in Collins and Grounds 2001). Our model may further differ from chronic inflammatory disorders with respect to the sustained levels of TNFα that may deplete the applied antibodies and, thus, more frequent injections of Remicade may be required for effective blockade in vivo.

Of particular interest, the inhibitory effects of both Remicade and Enbrel are apparent during the early stages of the acute inflammatory response (up to 5 days after grafting) but do not prevent the subsequent infiltration of inflammatory cells (at 7 days or later) and longer-term regeneration. One explanation is that the up-regulation of other cytokines compensates for the absence of functional TNFα in this experimental set-up. Such up-regulation is a feature of cytokine networks. This is certainly the most likely explanation for the lack of any impaired inflammation or adverse effects on regeneration for whole muscle grafts in mice genetically engineered to lack TNFα (Collins and Grounds 2001) in which other pro-inflammatory cytokines, such as interleukin-12 (IL-12) and interferon γ, may compensate for the TNFα deficiency. A similar cytokine redundancy probably accounts for the relatively disappointing results seen in mdx mice that lack TNFα (Spencer et al. 2000; Grounds and Torrisi 2004).

The reduced breakdown of dystrophic muscle in mdx mice treated with Remicade (Grounds and Torrisi 2004) or with Enbrel (S. Hodgetts et al., under review) further confirms the efficacy of these drugs in mice and indicates that these highly specific anti-inflammatory drugs may represent a useful clinical intervention to treat DMD and other myopathies with a pronounced inflammatory component, such as the dysferlinopathies (Hoffman et al. 2002; Bansal et al. 2003). The high specificity of Remicade and Enbrel appears advantageous compared with the existing use of anti-inflammatory corticosteroids, such as Prednisolone and Deflazacort, for treatment of DMD, as these steroids are associated with severe adverse side effects, such as weight gain and osteoporosis (Bushby et al. 2004); steroids should, in any case, be avoided for the dysferlinopathies because of the non-recoverable loss of strength (Hoffman et al. 2002). Clearly, many issues have to be considered with respect to the clinical applications of novel anti-cytokine therapies, which have yet to be evaluated for various myopathies. Other anti-inflammatory cytokine therapies include (1) a soluble receptor to IL-1, Anakinra (Kineret), which directly antagonises IL-1 via a similar principle to Enbrel and which is in clinical use (Fleischmann et al. 2003; Kary and Burmester 2003), (2) improved receptor strategies with cytokine traps (Economides et al. 2003), (3) the blockade of IL-15 and IL-18 that normally elevate TNFα (Canetti et al. 2003; McInnes et al. 2003) and (4) a host of other drugs targeted at downstream signalling and other consequences of elevated TNFα. Optimising the administration of such novel therapies requires suitable in vivo experimental models. The histological examination of whole muscle autografts at 5 days after transplantation in mice provides a simple reproducible model for assessing the ability of many novel therapeutic agents to reduce inflammation in vivo for potential application to mouse models of human disease.

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