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Immunology

Systemic immunological effects of cytokine genes injected into skeletal muscle

(immune modulation/gene delivery)

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ABSTRACT **Somatic gene therapy is an interesting approach for the delivery of cytokines for prolonged periods. The present experiments show that direct injections into mouse skeletal muscle of cDNA expression vectors encoding interleukin 2 (IL-2), IL-4, or type β1 transforming growth factor (TGF-β1) induce biological effects characteristic of these cytokines *in vivo*. Mice injected intramuscularly with a vector encoding IL-2 had enhanced humoral and cellular immune responses to an exogenous antigen, transferrin, that was delivered at a separate site. These IL-2 effects were abolished by coadministration of a vector directing synthesis of TGF-β1. The TGF-β1 vector by itself depressed the anti-transferrin antibody response and caused an 8-fold increase in plasma TGF-β1 activity. The TGF-β1 plasmid injection did not cause muscle infiltration with monocytes or neutrophils and there was no evidence for fibrotic changes. Muscle injection with a cDNA encoding IL-4 selectively increased IgG1 levels but did not alter the cellular immune response to transferrin. In lupus-prone mice (MRL/lpr/lpr), injection with IL-2 expression vectors increased and TGF-β1 vectors decreased autoantibodies to chromatin. These results demonstrate that intramuscular injection of cytokine genes, in the absence of infectious viral vectors, can regulate humoral and cellular immune responses *in vivo*.**

Cytokines and related peptide growth factors are being used therapeutically with increasing success. However, their applications have been limited, because of the necessity to administer relatively large amounts of recombinant proteins that usually have a short half-life. Somatic gene therapy is a potential approach to overcome this problem. Recent experiments have demonstrated the functionality of genes inserted into myoblasts. Three expression strategies have been used: (*i*) transplantation of transfected myoblasts (1, 2), (*ii*) viral vectors (3), and (*iii*) direct intramuscular (i.m.) plasmid cDNA injection (4, 5).

The advantages of direct DNA injection into skeletal muscles of living animals are the simplicity and safety of the technique. It can be accomplished without the use of infectious virus and without the need for integration of the gene of interest into the host genome. In addition, the level and persistence of expression obtained in some studies suggests that functional genes may be administered repetitively in a "drug-like" manner. However, this strategy has not yet been shown to produce biologically active growth factors *in vivo*, and there is no information to support whether or not local DNA injection can result in systemic effects with potential therapeutic significance.

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This study was designed to test the possibility that skeletal muscle can be used to express biologically active cytokines after injection with the analogous plasmid cDNA(s). The results demonstrate that injections of genes encoding interleukin 2 (IL-2), IL-4, and type β1 transforming growth factor (TGF-β1) induce systemic immunological effects that are specific functions of the respective cytokine proteins.

MATERIALS AND METHODS

Construction of Expression Vectors for IL-2, TGF-β1, and IL-4. The cDNAs for human IL-2 (ATCC 67618), TGF-β1 (ATCC 59954), and mouse IL-4 (ATCC 37561) were subcloned into the vector pBSII SK to generate 5′ *Hin*dIII and 3′ *Sma* I sites. These were used to replace the *Hin*dIII/*Bam*HI luciferase cDNA fragment in the expression vector pRSVL (4). The resulting expression vectors (pRSVIL2, pRSVTGFβ1, and pRSVIL4) contain the Rous sarcoma virus long terminal repeat promoter, and simian virus 40 polyadenylylation site. Plasmid DNA was purified from transformed DH5α *Escherichia coli* with Qiagen kits (Chatsworth, CA).

The activities of the three vectors were confirmed by transient lipofection of mouse C2C12 myoblasts (ATCC CRL 1772) as described (6). In each case, supernatants obtained 48 hr after lipofection contained biologically active cytokine, as determined by the lymphocyte-activation factor assay, using murine C3H/HeF thymocytes, in the presence or absence of the appropriate specific neutralizing antibodies (R&D Systems, Minneapolis).

Experimental Design. Five-week-old BALB/c mice were purchased from The Jackson Laboratory. At 6 weeks of age (day 0) animals were divided into four groups of four mice. At days 0, 7, and 14, groups 2–4 were injected i.m. with a 28-gauge needle at five different sites in the right thigh with a total of 100 μg of plasmid DNA (pRSVIL2, pRSVTGFβ1, or pRSVIL4) dissolved in 100 μl of normal saline. Group 1 was injected with 100 μl of normal saline. At days 3, 10, and 17 (3 days after the cytokine gene injections), all animals were immunized with 100 μg of keyhole limpet hemocyanin (KLH) (Sigma), dispersed in 100 μl of Imject Alum (aluminum hydroxide; Pierce) i.m. in the same thigh. The second set of experiments followed a similar protocol. Five groups of eight mice each were injected in the right thigh with 100 μg of plasmid DNA (pRSVIL2, pRSVTGFβ1, or pRSVIL4; groups 2–4, respectively). Group 1 was injected with normal saline, while group 5 was injected with 100 μg of pRSVIL2 and 100 μg of pRSVTGFβ1. In contrast to the first experiments, the antigen human transferrin (Sigma) was injected under the same conditions i.m. in the left shoulder. At day 56 or 63,

Abbreviations: IL, interleukin; TGF-β, type β transforming growth factor; KLH, keyhole limpet hemocyanin; DTH, delayed-type hypersensitivity.

mice were injected s.c. with 50 μg of antigen (KLH or transferrin, respectively) suspended in 50 μl of normal saline. All animals were bled weekly from the retroorbital plexus for measurement of antibody levels. Six-week-old MRL/lpr/lpr mice (The Jackson Laboratory) were injected three times at 4-week intervals, following the same method with 100 μg of pRSVIL2, pRSVTGFβ1, or pRSVnull (10 mice per group). Mice were bled at 16 weeks, 2 weeks after the last injection, for measurement of anti-chromatin antibody levels. In the transferrin experiment, three mice from the pRSVTGFβ1 group, one mouse from the control group, and one mouse from the pRSVIL2 group, died during bleeding or anesthesia.

Antibody Assays. Wells of microtiter plates (Costar 3590) were coated with human transferrin [100 μl per well; 10 μg/ml in borate-buffered saline, pH 8.0 (BBS), overnight] washed with the same buffer, and quenched with a 1% bovine serum albumin (BSA) solution in BBS. After washing twice in BBS/0.5% Tween 20, serum samples diluted 1:1000 in phosphate-buffered saline, pH 7.4 (PBS), were added to duplicate wells. After overnight incubation at 4°C, the plates were washed with BBS/Tween and incubated with biotinylated anti-mouse IgG and IgM (Jackson Laboratories, West Grove, PA) diluted 1:8000 in PBS. After 1 hr of incubation with peroxidase-labeled streptavidin (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted to 1:2000 in PBS containing 1% BSA, the plates were washed four times with BBS/Tween and incubated with TMB peroxidase substrate (Kirkegaard and Perry Laboratories). Thirty minutes later, absorption at 450 nm was measured in a Titertek multiscan meter (Flow Laboratories). Each assay mixture included a standard mouse anti-KLH or anti-transferrin antiserum that was diluted serially starting at 1:5000. In the transferrin experiment, the absorption values were converted to relative antibody concentrations. In the description of the results, 1 is defined as the antibody level in a 1:5000 dilution of the standard antiserum.

Total IgG and IgG1 concentrations were determined in the transferrin experiment with radial immunodiffusion kits (The Binding Site, San Diego) according to the manufacturer's instructions.

Antibodies to chromatin were assayed by ELISA as described (7). The ELISA absorbance values are referred to a standard curve that was established with a strongly positive reference serum. The results are the dilution of the standard curve, which gave the same absorbance as the test sera × 10^6, and have been shown to represent a linear measure of the amount of antibody present. The absolute units are arbitrary and are expressed as equivalent dilution factors.

Measurement of Circulating Levels of TGF-β1 Protein. To determine whether gene administration leads to a sustained increase in circulating cytokine levels, mice were bled at week 6, 4 weeks after the last gene injection, and the plasma samples were assayed for TGF-β1 activity by the CCL64 mink lung cell proliferation assay, slightly modified as described (8). Rabbit antibody that specifically neutralizes TGF-β1, but not TGF-β2 or TGF-β3, was purchased from R&D Systems (Minneapolis).

Measurement of Delayed-Type Hypersensitivity (DTH) Responses. To determine whether injection of cytokine genes modulates cellular immunity, DTH responses were tested by footpad swelling 48 hr after antigen challenge. At day 70, 100 μg of transferrin in 100 μl of normal saline was injected into the right hind footpad. Footpad thickness was measured with calipers before and 48 hr after injection, and the difference between these measurements was calculated.

Histological Evaluation. At day 91, three mice from each group of the transferrin experiment were sacrificed. Muscles into which the genes had been injected were fixed in 10% formalin and processed for histological evaluation.

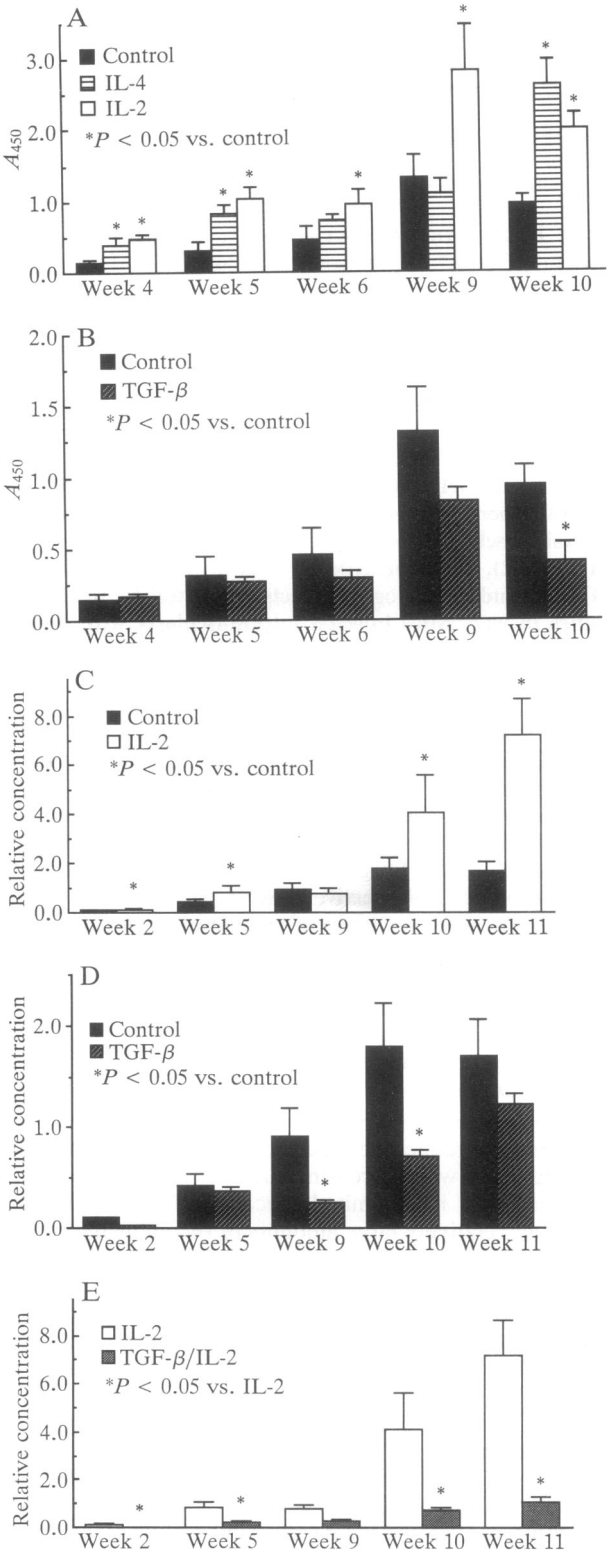

FIG. 1. Effects of cytokine gene injections on antibody responses. Groups of four BALB/c mice were injected i.m. on days 0, 7, and 14 with expression vectors for IL-2 and IL-4 (*A*) or TGF-β (*B*). On days 3, 10, and 17, animals were immunized at the same site with KLH. Following the same time schedule of injection, groups of eight mice were injected with expression vectors for IL-2 (*C*), TGF-β1 (*D*), or IL-2 plus TGF-β1 (*E*). However, here the animals were immunized at a separate site with human transferrin. Antibody responses were measured by ELISA at the times indicated on the abscissa. Anti-transferrin antibody results are expressed as mean relative antibody concentrations ± SEM compared to a standard antiserum.

Table 1. Effects of i.m. cytokine gene injections on total IgG levels following immunization with transferrin

	Week 0	Week 5	Week 10	Week 11
Control	1716 ± 72	4863 ± 234	7,475 ± 627*†	10,182 ± 578*
TGF-β	1956 ± 165	4404 ± 419†	7,456 ± 232*†	9,732 ± 528*
IL-2	1907 ± 55	5085 ± 168	10,977 ± 790	12,146 ± 348
IL-4	1828 ± 41	5572 ± 434	10,372 ± 982	11,651 ± 553
TGF-β/IL-2	1892 ± 101	5197 ± 124	8,330 ± 545*†	9,817 ± 872*†

Total IgG levels were determined by radial immunodiffusion. Values are shown as mg/liter and represent means ± SEM.
*$P < 0.05$ vs. IL-2.
†$P < 0.05$ vs. IL-4.

Statistical Analysis. Data from each experiment were compared by one factorial ANOVA.

RESULTS

Effects of Cytokine Gene Injection on Antibody Responses to Foreign Antigens. In the first set of experiments, we determined the effects of i.m. cytokine gene injections on antibody responses to a foreign antigen KLH that was injected at the same site as the plasmid. Antibodies to KLH reached higher levels in the pRSVIL2 and pRSVIL4 groups than in the control group (Fig. 1*A*). In contrast, anti-KLH antibodies were lower in the pRSVTGFβ1 group than in the control group (Fig. 1*B*). To determine whether the cytokine genes exerted their effects systemically, and not only at the regional lymph node, a second set of experiments was performed in which antigen (transferrin) and cytokine vectors were injected at two different sites. Antibodies to transferrin were highest in the pRSVIL2 group (Fig. 1*C*) and lowest in the pRSVTGFβ1 group (Fig. 1*D*). These results showed that injection of the two plasmids was capable of stimulating or inhibiting the antibody response. In several *in vitro* systems, TGF-β can antagonize the effects of IL-2. It is unknown whether TGF-β can have similar activities *in vivo*. Experiments were thus performed in which plasmids encoding IL-2 or TGF-β1 were injected simultaneously. Anti-transferrin antibody levels in the group that received both pRSVIL2 and pRSVTGFβ1 were indistinguishable from the pRSVTGFβ1 group (Fig. 1*E*), demonstrating that TGF-β1 expression completely neutralized the IL-2 effect. Mean anti-transferrin antibody levels in the pRSVIL4 group were higher but not significantly different from the control group (2.3 ± 0.4 vs. 1.7 ± 0.36 mg/liter in the 11th week). In control experiments, injections of the pRSV plasmid without any cytokine insert had no consistent effect on antibody levels (results not shown). Thus, the immunologic consequences of the cytokine gene injections cannot be attributed to nonspecific effects of DNA on interferon levels or to trace contamination with endotoxin.

Levels of Total IgG and IgG1. Levels of total IgG were measured in the sera from the transferrin-injected mice. The highest IgG levels were observed in the pRSVIL2 and the pRSVIL4 groups after 10 and 11 weeks; the lowest levels were detected in the pRSVTGFβ1 group (Table 1). TGF-β1 plasmid injections completely inhibited the IL-2-mediated increase. The levels of total IgG in the pRSVIL2/pRSVTGFβ1 group were similar to those in the pRSVTGFβ1 group. The mice injected with pRSVIL4 had significantly higher concentrations of IgG1 than the other groups (Table 2).

DTH. To determine whether cytokine gene injections can modulate cellular immunity, DTH responses were tested by a footpad swelling assay after antigen challenge. As shown in Fig. 2, there was a highly significant increase in footpad swelling in pRSVIL2-injected mice, as compared to the control group. Coinjection of pRSVTGFβ1 completely inhibited the IL-2 effect.

Levels of Circulating TGF-β1. The mean plasma levels of TGF-β1 at week 6, 4 weeks after the last pRSVTGFβ1 injection, were 2.6 ng/ml as compared to only 0.32 ng/ml in the pRSVIL2-injected or untreated animals, which represents an 8-fold difference. TGF-β activity was neutralized by specific antibodies to TGF-β1 (Table 3).

Histological Evaluation. Sections from muscles that had been injected with plasmid DNA showed multiple discrete loci of lymphocyte and macrophage infiltration in the pRSVIL2 and the pRSVIL4 groups (results not shown). In contrast, there was no muscle infiltration with monocytes or neutrophils in the pRSVTGFβ group, nor were there any detectable signs of fibrosis.

Anti-Chromatin Antibodies. The results from the transferrin studies showed that plasmid DNA injections induced biological effects characteristic of the three cytokines during cellular or humoral immune responses to a foreign antigen. To determine whether this approach is also effective in modulating ongoing pathological immune responses, we tested the IL-2 and TGF-β1 plasmids in MRL/lpr/lpr mice, which produce high titers of autoantibodies and are used as a model of systemic lupus erythematosus. Titers of autoantibodies to chromatin in the MRL/lpr/lpr mice were significantly increased in the pRSVIL2 group. The lowest titers were observed in the pRSVTGFβ1 group. The mean difference between the two groups was almost 7-fold (Fig. 3).

DISCUSSION

Gene expression in muscle tissue following injection of plasmid cDNA has been demonstrated previously in experiments using reporter gene constructs (1, 2, 4). However, the results presented here demonstrate that direct injection of

Table 2. Injection of IL-4 gene increases IgG1 levels following immunization with transferrin

	Week 5	Week 10	Week 11
Control	1320 ± 75 (0.27)*	2401 ± 121 (0.32)*	3562 ± 167 (0.35)*
TGF-β	1466 ± 76 (0.33)*	2568 ± 252 (0.34)*	3642 ± 171 (0.37)*
IL-2	1570 ± 95 (0.31)*	2797 ± 153 (0.26)*	3984 ± 163 (0.33)*
IL-4	2352 ± 207 (0.42)	4033 ± 302 (0.39)	4865 ± 343 (0.42)

IgG1 levels were determined by radial immunodiffusion. Values are shown as mg/liter and represent means ± SEM. Numbers in parentheses denote IgG1/IgG ratio at the same time point.
*$P < 0.05$ vs. IL-4.

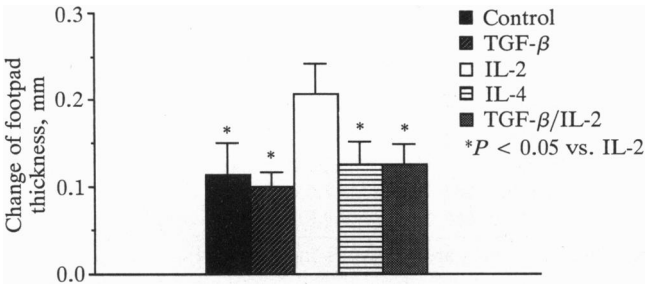

FIG. 2. Effects of cytokine gene injections on DTH responses. Animals in each group were challenged with 100 μg of transferrin injected in the right hind footpad. Footpad thicknesses were measured before and 48 hr after injection, and differences between preinjected and postinjected measurements were calculated. Results are expressed as means ± SEM.

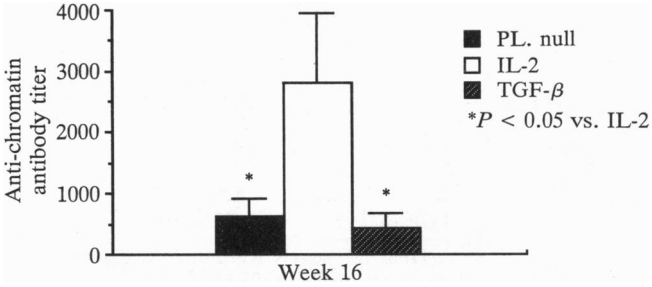

FIG. 3. Effect of cytokine gene injections on autoantibodies to chromatin. Groups of 10 MRL/lpr/lpr mice were injected i.m. every 4 weeks with pRSVIL2, pRSVTGFβ, or pRSVnull (PL. null; without any insert). Anti-chromatin antibodies were measured by ELISA at 16 weeks of age. Results are expressed as equivalent dilution factors.

cDNA expression vectors into muscle can produce biologically significant systemic effects. Cytokine genes and antigens were injected into experimental animals at different times and at disparate sites. Moreover, the immunologic actions of the cytokine genes persisted for weeks after administration. These results indicate that the effects of the cytokine genes were exerted systemically.

Injection of the IL-2 gene augmented the antibody response to immunization with KLH or transferrin, elevated total plasma IgG levels, and increased the magnitude of the DTH response in antigen-challenged animals. These results demonstrate that IL-2 gene injection can reproduce the central role of IL-2 in T-cell-dependent immune responses (9). Injection of the IL-4 gene increased total IgG levels and selectively upregulated IgG1 concentrations (10) but did not intensify the DTH response to antigen challenge (11). These effects are consistent with the established role of IL-4 in stimulation of immune responses mediated by the TH2 lymphocyte subset (12).

TGF-β1 is a multifunctional cytokine that generally antagonizes the actions of IL-2 (13). Injection of the TGF-β1 gene inhibited the humoral and cellular immunostimulatory effects of the IL-2 gene (14). Similarly, mice injected with the TGF-β1 gene alone had reduced antibody responses to transferrin, as compared to controls, presumably due to its immunosuppressive effects on B and T cells (15–18).

These data demonstrate that cytokine gene injections can successfully modulate immune responses to foreign antigens. This can be achieved in a stimulatory or inhibitory fashion, as shown for IL-2 and IL-4 vs. TGF-β1, respectively. Moreover, simultaneous administration of TGF-β1 can completely inhibit the immunostimulatory effects of IL-2 and IL-4. The same pattern of results was observed in the MRL/lpr/lpr mice. Injection of the IL-2 gene increased dramatically the level of anti-chromatin antibodies, while injection of the TGF-β1 gene reduced the level of this autoantibody.

Table 3. Circulating TGF-β activity

Plasmid	Total TGFβ activity	TGF-β1 activity
pRSVTGFβ1	2.6 ± 1.08	2.6
pRSVIL2	0.32 ± 0.11	0.32

Plasma samples from mice injected with either pRSVTGFβ1 or pRSVIL2 were collected 4 weeks after the last plasmid injection. Samples were diluted 1:10, acidified to pH 4, neutralized, and tested in triplicate in the CCL64 assay for TGF-β activity. Aliquots of the samples were also incubated with neutralizing rabbit antibody specific for TGF-β1 (10 ng/ml) before their addition to the CCL64 assay mixture. Preimmune rabbit IgG did not change the levels of TGF-β activity. TGF-β levels were derived by comparison with a standard curve containing recombinant TGF-β1 (R&D Systems), are shown in ng/ml, and represent means ± SE from three samples per group.

Cytokines are often difficult to detect in serum, due to their labilities, to their short half-lives, and to the relative insensitivity and imprecision of the available detection systems (19–21). Despite these difficulties, we detected an 8-fold increase in TGF-β1 concentrations in BALB/c mice 4 weeks after the last administration of the TGF-β1 gene.

TGF-β is secreted by most cell types in a latent precursor form, which can be activated enzymatically or by exposure to an acidic or basic milieu. The TGF-β1 construct used in the present studies encoded the precursor protein, and, consistent with this, we detected only latent TGF-β in cells transfected with this vector *in vitro* or in the plasma of mice receiving i.m. injections. However, TGF-β inhibited cellular and humoral immune responses when injected into muscles distinct from the site of antigen injection. This suggests that TGF-β is activated in lymphoid tissues. There was no detectable evidence for local manifestations of TGF-β effects at the sites of plasmid injection. Specifically, we did not find muscle infiltration with monocytes or neutrophils or signs of fibrotic changes. The present results thus suggest that TGF-β can be administered by gene injection without inducing fibrosis and inflammatory cell infiltration, effects that would argue against the therapeutic use of TGF-β as an immunosuppressive agent.

Many different cytokines have been described in the past several years. The large number of different cytokines has made it difficult to ascertain their respective physiologic roles and to determine how they interact to regulate immune and inflammatory responses. Intramuscular injection of expression vectors directing the synthesis of each of these cytokines may provide a simple and powerful method to address this problem. It obviates the need to express, purify, and administer multiple different recombinant cytokine proteins. Moreover, this approach should facilitate the *in vivo* analysis of cytokine genes that have been changed in the laboratory to produce proteins with altered pharmacologic properties.

Several potential clinical applications of cytokine gene therapy can be proposed. The production of TGF-β1 or IL-10, which is a potent immunosuppressant of monocyte function (13), by transfected skeletal muscles may help to control severe autoimmune and inflammatory diseases. The coadministration of particular combinations of cytokine genes may be able to modulate the immune response to vaccination by favoring the formation of cytotoxic T cells versus neutralizing antibodies. Secretion of IL-2 may be efficacious in immune deficiency states or in patients with tumors (22). In conclusion, the present results suggest that expression of biologically active cytokines, following plasmid injection, and the resulting modulation of cellular responses provide a basis for systemic delivery of biologically active proteins in a wide spectrum of immunologic diseases.

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