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Research Article

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Immunomodulation by Mucosal Gene Transfer Using TGF- β DNA

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Abstract

This report evaluates the efficacy of DNA encoding TGF- β administered mucosally to suppress immunity and modulate the immunoinflammatory response to herpes simplex virus (HSV) infection. A single intranasal administration of an eukaryotic expression vector encoding TGF- β_1 led to expression in the lung and lymphoid tissue. T cell-mediated immune responses to HSV infection were suppressed with this effect persisting as measured by the delayed-type hypersensitivity reaction for at least 7 wk. Treated animals were more susceptible to systemic infection with HSV. Multiple prophylactic mucosal administrations of TGF- β DNA also suppressed the severity of ocular lesions caused by HSV infection, although no effects on this immunoinflammatory response were evident after therapeutic treatment with TGF- β DNA. Our results demonstrate that the direct mucosal gene transfer of immunomodulatory cytokines provides a convenient means of modulating immunity and influencing the expression of inflammatory disorders. (*J. Clin. Invest.* 1998. 102:438–444.) Key words: gene transfer • mucosal • therapeutic • suppress • inflammation

Introduction

It has become evident that the administration of plasmid DNA expression vectors represents a novel means of vaccination which could replace some existing vaccines and may even provide an immunization approach for some agents currently lacking effective vaccines (1). More recently, we and others have also shown that the DNA expression vector approach also represents a method of achieving cytokine expression and this may serve to modulate vaccine immunogenicity as well as influence the extent of immunoinflammatory lesions (2). In this report, we have investigated if the DNA expression vector approach provides a useful method of expressing an active form of TGF- β_1 . TGF- β_1 was chosen since this cytokine is the principal member of a family of proteins which have multiple effects on component cells of the inflammatory response. Indeed TGF- β knockout mice generate uncontrolled inflammatory responses in various organs. The production of TGF- β_1 which results from antigen stimulation of specific cells is sus-

pected to provide an explanation for oral tolerance (3, 4), and the induction of such unresponsiveness is being explored as a means of controlling certain immunoinflammatory diseases (5, 6). More direct evidence for the potential value of TGF- β_1 to suppress inflammatory disease has come from model animal studies in which the recombinant protein was used for treatment. Prophylactic administration was claimed effective to suppress experimental allergic encephalomyelitis, inflammatory bowel disease, as well as uveitis (3, 7–10). However, effective therapy required repeated administration of substantial quantities of protein (7). In this report, we show that only a single administration of TGF- β_1 DNA is sufficient to cause marked suppression of antigen-specific T cell responses. Moreover, the TGF- β_1 DNA was administered via the noninvasive mucosal route. Suppressed animals became more susceptible to vaginal infection by herpes simplex virus (HSV).¹ In addition, mucosal TGF- β_1 DNA administration was effective when used prophylactically to modulate the severity of ocular immunoinflammatory disease resulting from HSV infection. Our results demonstrate the potential clinical value of using expression plasmids encoding proteins to influence inflammatory reactions and demonstrate that the noninvasive nasal route may provide a convenient means of DNA delivery.

Methods

Mice. Female BALB/c mice (H2^d) purchased from Harlan Sprague-Dawley (Indianapolis, IN) were used for all experiments. The investigators adhered to guidelines proposed by the Committee on the Care of Laboratory Animals Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee are fully accredited by the AALAC.

Virus. HSV-1 (McKrae), HSV (KOS), or (RE) was grown on Vero cell monolayers (ATCC CCL81) and was stored in aliquots at –80°C until used. Titers were measured in Vero cells and expressed as pfu per milliliter (11).

Plasmid preparation. Plasmid DNA encoding TGF- β or IL-2 under the control of a CMV promoter were gifts of Dr. Gundie Ertl (Philadelphia, PA) and plasmid encoding IFN- γ with the same promoter was provided by Dr. Shau-Ku Huang (Johns Hopkins Asthma and Allergy Center, Baltimore, MD). Plasmids were purified by polyethylene glycol precipitation according to Sambrook et al. (12) with some modifications. Cellular proteins were precipitated with 1 vol of 7.5 M ammonium acetate followed by isopropanol precipitation. Plasmids were phenol-chloroform extracted (two times) and precipitated with 2 vol of pure ethanol. The quality of DNA was checked by electrophoresis on 1% agarose gels.

Plasmid administration. 20–30 μ l of PBS containing 100 μ g of plasmid DNA encoding cytokines was administered intranasally (i/nas) to mice anesthetized with metophane (methoxyflurane; Pittmann-Moore, Inc., Mundelein, IL).

HSV infection. Mice given plasmid DNA encoding cytokines i/nas were infected 7 d later with 10⁶ pfu of live HSV by the same

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1. **Abbreviations used in this paper:** CLN, cervical lymph node; DC, dendritic cell; DTH, delayed-type hypersensitivity; HSK, herpetic stromal keratitis; HSV, herpes simplex virus; i/nas, intranasal; MLN, mediastinal lymph nodes; SFC, spot-forming cell.

route. 15 d later, serum was collected for antibody measurement. The mice were killed and the splenocytes were collected for immunological analysis. In some experiments, mice were infected with virus intraocularly as described elsewhere (2).

Ocular infection and clinical observation. BALB/c mice were given 100 µg of plasmid DNA encoding cytokines *i/nas* on three occasions (days 0, 7, and 14). On day 15 the mice were anesthetized, and the corneas were scarified as described previously (2). HSV-1 RE (10⁶ pfu in 4 µl volume) was administered to the scarified cornea and gently massaged with the eyelids. Animals were examined daily after infection and severity of the stromal keratitis was graded 0 to 5 as described elsewhere (2): 0, clear eye; 1, local or mild limbal neovascularization, mild corneal haze; 2, abundant neovascularization that invaded to the central cornea, moderate cell infiltration; 3, severe corneal opacity, iris vessel engorgement; 4, opaque cornea, iris not visible; and 5, complete corneal rupture and necrotizing stromal keratitis.

HSV-specific proliferative assay. To determine if HSV-specific T cell responses were affected by TGF-β DNA administration, splenic and cervical lymph node (CLN) lymphoproliferative responses were measured and compared with responses in control mice which received either IL-2 DNA or IFN-γ DNA. Splenocyte proliferative responses were measured 15 d after *i/nas* HSV infection and CLN 5 d after infection. Splenic cells were stimulated with HSV-1 KOS (moi of 1.5 before UV inactivation). The HSV-specific lymphoproliferation was measured as described elsewhere (13). Cultures were maintained for 5 d and proliferative responses expressed as cpm ±SD of five replicates per sample. All cultures were additionally stimulated with ConA at 5 µg/ml for 3 d.

Delayed-type hypersensitivity (DTH). This was measured as described elsewhere (13). Briefly, animals received 20 µl of PBS containing UV-inactivated HSV-1 (KOS). Ear thickness was recorded after 48 h with a screw gauge micrometer (Oditest; H.C. Kroepelin GmbH, Schluechtern, Germany) and recorded as mean increase in thickness. Each test group had at least 10 animals.

Cytokine ELISPOT. The method used was described in detail previously (14). In brief, splenocytes from test animals were analyzed for IL-4, IL-5, and IFN-γ spot-forming cells (SFCs). To generate cytokines, the splenocytes were stimulated with enriched dendritic cell (DC) populations obtained as described by Nair et al. (15), that had been pulsed 3 h before adding to responder splenocytes with UV-inactivated HSV (moi of 5 before UV inactivation). The responder splenocytes and stimulator DCs (naive or pulsed with UV HSV) were added at responder to stimulator ratios of 50:1, 25:1, 12.5:1, and 6.25:1 in 200 µl RPMI 10% FBS per well into coated and blocked ELISPOT plates. ELISPOT plates were precoated with anticytokine antibodies (anti-IL-5 Cat. No. 18051D, anti-IL-4 Cat. No. 18031D, and anti-IFN-γ Cat. No. 18111D; PharMingen, San Diego, CA) at a concentration of 2 µg/ml in 100 µl in sterile carbonate buffer at 4°C overnight and were subsequently blocked with RPMI 10% FBS media for 1 h at 37°C. After a 72-h incubation of the effector and stimulator cells (in a vibration-free incubator), the ELISPOT plates were washed with PBS (3×) and PBS-Tween 20 (PBST) (3×), and 100 µl of biotinylated anti cytokine antibodies (biotinylated anti-IL-5 Cat. No. 18062D, biotinylated anti-IL-4 Cat. No. 18042D, and biotinylated anti-IFN-γ Cat. No. 18112D; PharMingen) diluted 1:1,000 in PBST 1% FBS were added to the plates. After a 1-h incubation at 37°C, 100 µl of 1:1,000 diluted alkaline phosphatase-conjugated streptavidin in PBST 1% FBS was added and the plates were incubated for another hour at 37°C. The spots were developed using the substrate [NBT], [BCIP], and counted 24 h later using a dissecting microscope.

ELISA. Anti-HSV antibody levels were determined as described in detail elsewhere (14). Briefly, serum was tested for total IgG as well as for IgG_{2a} and IgG₁ level using ELISA plates coated with HSV antigen and goat isotype-specific antisera to detect mouse antibody binding. The system was quantified by running standard curves using Spectra Max ELISA reader softmax version 1.2.

IL-2 bioassay. This was described previously (13). Basically the approach used employed CTLL/2 cells (kindly supplied by Drs. Cyn-

thia Watson and William Paul, National Institutes of Health, Bethesda, MD), and compared the ability of test samples to support CTLL cell proliferation with that of standard known amounts of recombinant IL-2 (Cat. No. Cy-24-025; Biosource International, Camarillo, CA). The concentration of IL-2 in the samples was determined from a standard curve as previously described (13).

T helper cell frequency analyses. A limiting dilution assay was used to quantitate the frequency of T helper cells activated *in vivo* by HSV. Splenocytes from test animals were irradiated (2,000 rads) and seeded in replicates in serial twofold dilutions into 96-well round-bottomed plates as described by Slater et al. and Mohler and Butler (16–18). CTLL/2 cells were added to all wells containing spleen cells and additional control wells containing only media (negative control). Plates were incubated at 37°C and 5% CO₂ for 48 h. All wells were subsequently supplemented with 1 U/well IL-2 and incubated for an additional 4 d. CTLL/2 retaining viability from IL-2-producing splenic cells during the initial 48 h are greatly expanded, whereas the additional amount of IL-2 does not induce proliferation of IL-2-starved cells in control wells (16). CTLL/2 proliferation in these experiments was quantitated by [³H]thymidine incorporation as described above. Wells were scored as positive when counts per minute were greater than the mean plus three standard deviations of the negative control wells containing only CTLL/2 cells and media. Frequency values were determined by minimum χ² analysis (19).

Vaginal challenge. The model used was described elsewhere (14). To test whether *i/nas* plasmid DNA administration will result in increased susceptibility to HSV infection, naive cytokine-treated animals were challenged intravaginally with virulent HSV-1 (McKrae). Age-matched control mice given vector DNA *i/nas* served as controls. 7 d after *i/nas* administration of plasmid DNA (100 µg/mouse) the mice were injected subcutaneously with 2 mg/mouse of Depo Provera (Upjohn Co., Kalamazoo, MI) to synchronize the estrus cycle at a progesterone-dominated stage as described by Parr et al. (20). 5 d after hormone administration, the mice were infected intravaginally with 5 × 10⁴ pfu (1 × LD₅₀) of HSV-1 McKrae. These mice were examined daily for vaginal inflammation and death.

Viral titration. Vaginal washings were collected at different time points after intravaginal challenge by pipetting 100 µl of PBS into the vaginal cavity. The samples were stored at -80°C until used. Individual samples (50 µl from each sample) were further diluted and viral titers were obtained using a plaque assay performed on Vero cells as described elsewhere (11).

Detection of TGF-β plasmid DNA. After intranasal administration of TGF-β or vector DNA, different organs including lung, mediastinal lymph nodes (MLN), spleen, and CLN were removed at intervals. Tissues were homogenized and added to Tri-reagent (Molecular Biology Inc., Cincinnati, OH) to isolate plasmid DNA. The supernatant containing the plasmid DNA was ethanol-precipitated, the DNA pellet was free of genomic DNA as verified by PCR for β-actin as well as electrophoresis on 0.8% agarose after digestion by different restriction enzymes. A 339 bp of plasmid DNA was amplified by PCR (21) using specific primers corresponding to the COOH-terminal sequence of the murine TGF-β1 cDNA: 5':GCC CTG GAT ACC TAT TGC and 3':GCT GCA CTT GCA GGA GCG CAC.

β-galactosidase (β-gal) detection. Lung, MLN, and spleen were collected at different times after intranasal administration of TGF-β or vector DNA. Tissues were fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO) in PBS for 1 h at 4°C and then placed overnight in X-gal substrate solution (2). Tissues were embedded in paraffin, sectioned at 5 µm, and then stained with nuclear fast red.

Statistics. The values obtained from different experiments were analyzed for statistical significance by Student's *t* test.

Results

Pattern of transduced gene expression after mucosal delivery of plasmid DNA. After *i/nas* administration of TGF-β DNA or

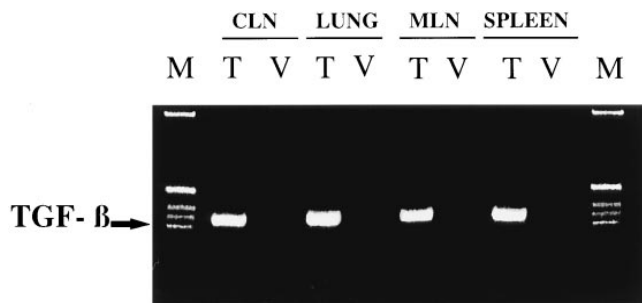


Figure 1. Detection of TGF- β plasmid DNA in CLN, lung, MLN, and spleen. Tissues were removed 5 d after intranasal administration of TGF- β or vector plasmid DNA. The presence of plasmid encoding TGF- β was assessed by PCR using specific primers. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. M, 1-kb DNA ladder; T, TGF- β DNA-administered tissues; V, vector DNA-administered tissues.

β -gal DNA to BALB/c mice, animals were killed at intervals after exposure and various organs were analyzed for the presence of transduced plasmid DNA or protein expression. For protein expression, an exogenous marker, β -gal, was used to avoid any confusion between endogenous TGF- β and the TGF- β derived from DNA *i/nas* administered. As shown in

Fig. 1, plasmid DNA encoding TGF- β was detected in several tissues including some distance (e.g., spleen) from the *i/nas* site of plasmid deposition. As shown in Fig. 2, protein expression was detected by histochemistry in tissues after *i/nas* administration of β -gal DNA. The administered DNA and protein expression was evident as soon as 1 d after injection and was still demonstrable at 30 d after the single *i/nas* exposure. Cell types expressing β -gal in the lung appeared to be epithelial cells as well as alveolar macrophages. Histopathological analysis of lungs at 5 and 30 d after TGF- β DNA administration revealed no evidence of morphologic changes.

Modulation of immune responses by mucosal administration of TGF- β DNA. Animals were given a single *i/nas* administration of cytokine or vector DNA and 7 d later infected by the same route with an immunizing dose of nonvirulent HSV. The effects on immune responsiveness to HSV were subsequently measured. The results recorded in Tables I and II indicate that animals which received TGF- β *i/nas* had markedly suppressed immune responses compared with those given either IL-2 or IFN- γ or vector DNA. Mainly cell-mediated immune responses were affected. Total HSV-specific IgG serum levels were similar. However, the ratio of IgG_{2a}/IgG₁ was decreased in TGF- β -treated animals as a result of enhancement of IgG₁ isotype (Table I). Thus the IgG_{2a}/IgG₁ ratio in TGF- β -treated animals was less than in other groups. It is to be noted that one sequel to TGF- β DNA administration was

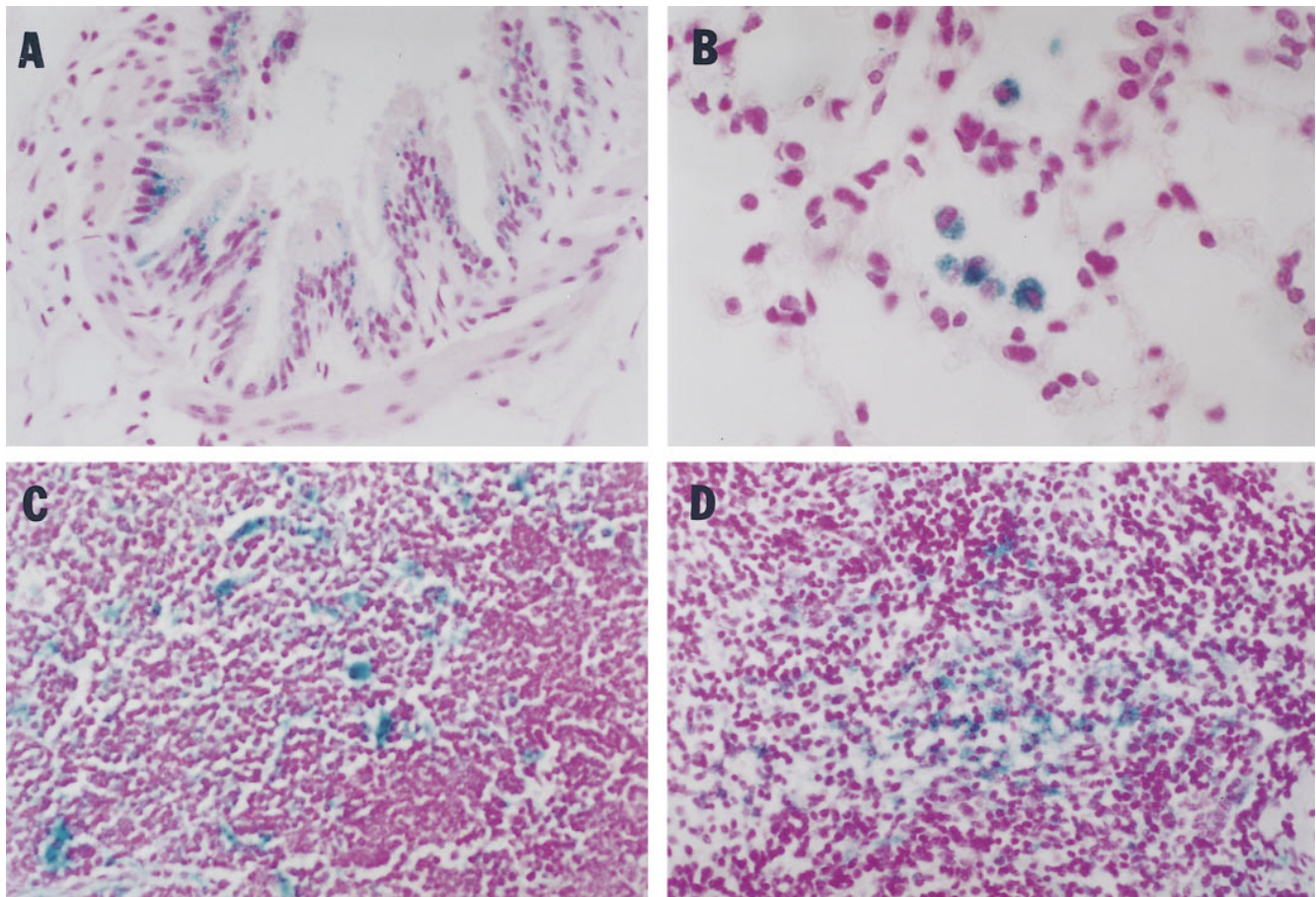


Figure 2. Expression of β -gal in different tissues 5 d after intranasal administration of plasmid DNA encoding β -gal. (A) Lung mucosal epithelium. (B) Alveolar macrophages. (C) MLN. (D) Spleen. $\times 250$.

Table I. Effect of Cytokine DNA Administered Intranasally on the Immune Response to HSV

Immune parameters	Tissues	Cytokine DNA			
		TGF- β	IL-2	IFN- γ	Vector
Lymphoproliferation*					
HSV	Spleen	465 \pm 168	26166 \pm 3814	15230 \pm 1480	23471 \pm 895
Unstim.		38 \pm 10	1415 \pm 854	315 \pm 118	483 \pm 170
Con A		35959 \pm 9830	26059 \pm 6725	25347 \pm 5260	19751 \pm 1110
HSV	Draining lymph node	63 \pm 11	276 \pm 82	584 \pm 125	670 \pm 93
Unstim.		34 \pm 16	33 \pm 10	41 \pm 10	38 \pm 11
Con A		4248 \pm 320	15921 \pm 450	17200 \pm 635	3105 \pm 435
Ag sp. ELISPOT [‡]					
IL-4	Spleen	22 \pm 2	38 \pm 9	ND	38 \pm 6
IL-5		10 \pm 3	26 \pm 9	ND	22 \pm 7
IFN- γ		37 \pm 7	554 \pm 40	ND	313 \pm 17
IL-10		12 \pm 3	1 \pm 1	ND	1 \pm 1
Bioassay for IL-2 [§]					
HSV	Spleen	108 \pm 30	9521 \pm 1675	ND	5966 \pm 1191
Con A		5253 \pm 633	12361 \pm 927	ND	10138 \pm 852
LDA					
IL-2		1:119305	1:30612	ND	1:22410
Ag. sp. antibodies [¶]					
IgG		51243 \pm 1800	59710 \pm 2218	59275 \pm 2107	48571 \pm 1980
IgG _{2a}		34439 \pm 18031	39180 \pm 9130	35677 \pm 13221	37116 \pm 18350
IgG ₁		6381 \pm 2491	1089 \pm 908	1038 \pm 406	2010 \pm 608
IgG _{2a} /IgG ₁		5.4	36.0	34.4	18.5

*HSV-specific proliferation assay. BALB/c mice were administered i/nas 100 μ g of plasmid DNA encoding cytokine. 7 d after immunization, the animals were infected with 10⁶ pfu of HSV-KOS by the same route. CLN and splenic lymphoproliferation responses were determined at days 5 and 15, respectively, after HSV infection as described in Methods. The data represent the mean counts per minutes \pm SD based on four independent experiments. [‡]ELISPOT assay. Splenocytes from mentioned above mice were in vitro restimulated for 3 d with naive or UV-HSV pulsed DC population. The number of SFCs was determined by ELISPOT assay. The number of cytokine SFCs after naive DC restimulation was subtracted from the number of SFCs obtained after HSV-pulsed DC restimulation. [§]Bioassay. The splenocytes were collected 15 d after HSV-1 infection and in vitro stimulated with 2 moi of HSV-1 or Con A for 72 h. Culture supernatants were harvested and screened for IL-2 using CTLL2 cells. The results are presented as pg/ml. ^{||}Limiting dilution assay. The frequency of IL-2-producing cells was measured using CTLL2 cells as described in Methods. [¶]Antigen (HSV)-specific antibodies. Serum samples were collected at 15 d after HSV-1 infection and tested for IgG, IgG₁, and IgG_{2a} HSV-specific antibody by ELISA. The data are presented in ng/ml and standard deviation is based on individual values (7–10 mice per group).

the upregulation of IL-10 expression in splenocytes as measured by the SFC assay.

The effect of TGF- β DNA administration on cell-mediated immune responses was particularly marked. Treated animals showed suppressed antigen-specific proliferative responses in both splenocyte and draining LN cells. In addition, measurement of cytokine-producing cell responses by the ELISPOT assay revealed decreased frequency of HSV-stimulated SFC for IL-4, IL-5, and IFN- γ . Measurements of HSV antigen-stimulated IL-2-producing cells by a limited dilution bioassay approach revealed marked inhibited responses in TGF- β DNA recipients. The suppressive effects resulting from TGF- β DNA administration were confined to antigen-specific responses since both splenocytes and draining lymph nodes responded almost normally to ConA stimulation.

As an additional measure of T cell immunity, cytokine-treated HSV-infected mice were also tested for DTH responses on two or more time periods after treatment. As is evident in Table II, DTH responses were markedly suppressed in TGF- β DNA-treated animals when measured both at 27 and 65 d after TGF- β DNA exposure (14 and 50 d after infection).

The finite duration of TGF- β DNA suppression was not established. Interestingly, there was no therapeutic effect of mucosal TGF- β DNA administration on the DTH reaction (Table II).

Modulation of immunity and immunopathology by mucosal cytokine delivery. The above analyses revealed that TGF- β DNA had broad suppressive effects on T cell-mediated immune responses to HSV infection. To evaluate the in vivo consequences of the immunosuppression, two approaches were used. In the first, the effects of TGF- β DNA administration on the resistance level of mice to vaginal challenge with a virulent strain of HSV were measured. A dose of virus was used which in previous experiments was shown to represent between 0.1 and 1 LD₅₀ (14). As is apparent in Table III, animals which received i/nas preexposure to TGF- β DNA were more susceptible to vaginal challenge than were recipients of vector DNA. Accordingly, TGF- β DNA recipients died more quickly and fewer animals survived the challenge (Table III).

In a second approach, to measure the in vivo consequences of TGF- β administration, we took advantage of the observation that HSV infection of the eye leads to the development of

Table II. Effect of Cytokine DNA Administered Intranasally on DTH Response to HSV

DTH	Cytokine DNA		
	TGF- β	IL-2	Vector
Prophylactic treatment*			
Day 14	5.6 \pm 2.0	ND	13.0 \pm 2.7
Day 20	5.5 \pm 2.9	10.0 \pm 1.0	15.0 \pm 3.5
Day 40	4.25 \pm 2.5	9.0 \pm 2.0	16.0 \pm 2.0
Day 50	5.2 \pm 2.1	13.1 \pm 1.0	13.5 \pm 2.0
Therapeutic treatment [‡]			
Day 40	10.8 \pm 2.5	ND	12.9 \pm 1.0

*BALB/c mice given 100 μ g of plasmid DNA encoding cytokine on day 0, followed by i/nas HSV administration (day 7), were subjected to DTH analysis on days 14, 20, 40, and 50 after infection. TGF- β DNA versus IL-2 DNA or vector DNA, all the time points, $P < 0.05$. [‡]Groups of HSV immune mice were given i/nas 100 μ g of plasmid DNA encoding TGF- β or vector, and DTH responses were measured 7 d after cytokine DNA administration (HSV, day 0; plasmid DNA, day 33; DTH, day 40). TGF- β DNA versus vector, $P > 0.1$. Values obtained from Vero extract-injected ears were subtracted from the values of HSV-injected ears. The data are presented as average increase in ear thickness measured at 48 h after challenge. SD is based on values obtained from 10 mice per group.

a blinding immunoinflammatory response termed herpetic stromal keratitis (HSK). This disease is known to be a T cell-mediated immunopathological lesion mainly involving CD4⁺ T cells of the Th1 cytokine-producing phenotype (21). To measure the effects on HSK, TGF- β DNA- and control vector-treated animals (on days 0, 7, and 14) were ocularly infected with the RE strain of HSV, 1 d after the last plasmid DNA exposure. Animals were followed clinically for the next 21 d and the severity of the HSK lesions was measured periodically with a biomicroscope. As is shown in Table IV, TGF- β DNA administration was effective at diminishing the severity of HSK. Thus, mean scores in 40 eyes were reduced from 4.7 \pm 0.2 to

Table III. Effect of TGF- β DNA Administration on Susceptibility to Vaginal Challenge with HSV

i/nas administration	Number of mice dead/total number of mice	Average time of death*	Virus titer [‡]	
			Day 2	Day 5
<i>d</i>				
TGF DNA	16/18 (88%)	7.8 \pm 0.7	4.7 \pm 0.7	4.1 \pm 0.6
Vector DNA	8/18 (44%)	10.7 \pm 1.1	4.9 \pm 0.4	2.7 \pm 1.0

BALB/c mice were given i/nas plasmid DNA encoding TGF- β . Age-matched mice were administered vector DNA by the same route and were used as controls. 2 d after plasmid DNA administration, the animals were injected subcutaneously with 2 μ g/mouse of DP. 5 d after hormone administration, the mice were intravaginally infected with 5×10^4 pfu of HSV-1 McKrae. This dose of virus was shown in previous experiments to represent ~ 1 LD₅₀ of virus challenge. *TGF- β DNA versus vector DNA, $P < 0.05$. [‡]Day 2, TGF- β DNA versus vector DNA, $P > 0.01$. Day 5, TGF- β DNA versus vector DNA, $P < 0.05$.

Table IV. Effect of Intranasal Delivery of Plasmid DNA-expressing Cytokines on the Severity of Ocular Inflammation

HSK score on day	Cytokine DNA		
	TGF- β *	IL-2 [‡]	Vector [§]
Prophylactic treatment			
9	0.7	1.5	1.7
15	2.1	3.0	4.7
20	3.1	4.7	4.6
Therapeutic treatment			
9	1.5	1.7	1.8
15	3.9	4.1	4.2
20	4.2	4.3	4.4

BALB/c mice were given i/nas 100 μ g of plasmid DNA cytokines at days 0, 7, and 14. 1 d after the last immunization, the cornea was scarified and HSV-1 RE (10^6 in 4- μ l volume) was placed on the cornea and massaged with the eyelids. The severity of stromal keratitis in each cornea was graded from 0 (clear eyes) to 5 (necrotizing stromal keratitis) as described in Methods. Each group consists of 40 eyes scored on days 9, 15, and 20 after infection. For therapeutic administration, scarified corneas were infected with HSV-1 RE and TGF- β DNA was administered at day 7 after infection. Prophylactic plasmid administration, day 9, * vs. [‡] or [§], $P < 0.05$; [‡] vs. [§], not statistically significant ($P > 0.05$); day 15, * vs. [‡] or [§], $P < 0.05$; [‡] vs. [§], not statistically significant ($P > 0.05$); day 20, * vs. [‡] or [§], $P < 0.05$; [‡] vs. [§], not statistically significant ($P > 0.05$). Therapeutic plasmid administration, all TGF- β -treated animals compared to vector-treated control group had $P > 0.05$.

3.1 \pm 0.4 ($P < 0.05$). Interestingly, none of the TGF- β DNA-treated animals succumbed to HSV encephalitis, perhaps an unexpected finding in light of the fact that treatment increased susceptibility to vaginal challenge with HSV (albeit a more lethal strain).

Discussion

In this report, we demonstrate that mucosal administration of plasmid DNA encoding the active form of TGF- β_1 , results in a marked suppression of T cell-mediated immunity that persists, as measurable by the DTH response, for several weeks. Suppression of several assays of mainly CD4⁺ T cell immunity was evident and animals became more susceptible to vaginal infection by HSV. In addition, the prophylactic administration of TGF- β_1 DNA diminished the severity of CD4⁺ T cell immunoinflammatory responses upon subsequent ocular infection with HSV. Our novel observations indicate that TGF- β_1 DNA administration via the noninvasive nasal route represents a convenient and innocuous means of delivering bioactive material. The approach could prove useful to manage immunoinflammatory disease where TGF- β_1 is known to have a beneficial effect. Thus, TGF- β_1 protein was observed to inhibit the expression of experimental allergic encephalomyelitis, inflammatory bowel disease, and uveitis (3, 7, 8). In all of these examples, which are mainly CD4⁺ T cell-mediated diseases, repeated administration of usually large doses of protein was needed to achieve an effect. Interestingly, suppressive effects may accrue from TGF- β_1 DNA administration after only a single exposure and this effect is surprisingly long-lasting.

Whereas useful antiinflammatory effects of TGF- β_1 DNA administration occurred when used prophylactically, therapeutic administration was without effect at least in the HSK model. In certain inflammation models, TGF- β_1 protein administration may have some therapeutic efficacy, but only if used early in the disease process (9). This requirement for prophylactic administration or early phase treatment to achieve antiinflammatory effects represents a shortfall for most clinical situations since therapy of overt lesions is the usual indication. However, it seems likely that better delivery systems for cytokine DNA, or the use of appropriate combinations of encoding DNA, may achieve higher efficacy. Currently, for example, we are testing the use of a liposome type delivery vehicle and using the oral route of exposure which appears to secure DNA expression after such delivery (22).

Although our results show a durable modulatory effect after mucosal delivery of TGF- β DNA, the actual mechanism by which the immunomodulation proceeds requires further investigation. We favor the notion that the effect occurs in lymphoid tissue and may result from effects on costimulation of T cells perhaps in part indirectly by upregulation of IL-10. Thus TGF- β was readily demonstrable in lymphoid tissue, and cells stimulated *in vitro* responded normally to T cell mitogens, but not to specific antigen stimulation. Such an effect could result if TGF- β downregulated IL-2R expression as has been reported to occur by others (23). Alternatively, TGF- β DNA could operate indirectly by causing alveolar macrophage proliferation in lungs with such cells exerting suppressive effects as has been observed in some systems (24). We did note plasmid expression in alveolar macrophages after mucosal delivery, but such cells were not increased in number in TGF- β recipients. Moreover, modulatory effects of TGF- β also occurred after intramuscular administration and protein expression was not evident in lungs after this route of administration (data not shown). We did observe, as reported by others (8, 25, 26), that TGF- β did cause the upregulation of another regulatory cytokine, IL-10. It remains possible that the effects we observed were mediated at least in part by such IL-10 upregulation, an idea also advocated by others (8, 25). Interestingly, IL-10 DNA can also cause inflammation suppression even when used early in the disease process, but this only applies after intraocular and not after *in*nas administration (Chun, S., and B.T. Rouse, unpublished data). Currently, we are testing if mucosal administration of combinations of TGF- β_1 and IL-10 DNA as well as certain other cytokine DNAs will suppress the expression of on-going HSK. The use of plasmid DNA-encoding cytokines is a potential clinical stratagem still in its infancy. Its strength lies in the convenience of the approach, the lack of expense, and the fact that the bioactive effects result from a single administration. Indeed, it could be that the expression plasmid approach will be a convenient means of achieving gene delivery to correct certain genetic defects. However, the observation that expression may be long lasting also represents a potential problem. Unwanted side effects may result. Thus, as was shown in this communication, although TGF- β_1 modulated HSV-induced immunoinflammatory disease, it also made animal recipients more susceptible to infection. Thus, it will probably be necessary to control cytokine DNA expression and to terminate expression upon demand. Whether or not such an effect is achievable with plasmids which use manipulable promoters is one such approach under investigation.

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