Distribution Fate and Mechanism of Immune Modulation Following Mucosal Delivery of Plasmid DNA Encoding IL-10

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Distribution Fate and Mechanism of Immune Modulation Following Mucosal Delivery of Plasmid DNA Encoding IL-10

Sangjun Chun, Massoud Daheshia, Sujin Lee, Seong Kug Eo, and Barry T. Rouse

DNA vaccination has been widely studied in several models of vaccination and in the treatment of inflammatory diseases, even though the mechanism involved is still unclear. This report demonstrates that mucosal administration of plasmid DNA leads to rapid and widespread distribution around the body. Dissemination likely occurred via the bloodstream because plasmid DNA was present in blood plasma. The plasmid DNA was also detectable in several tissues including draining lymph nodes, spleen, liver, bone marrow, and even the dermis of ear pinnae. Except for the site of administration, plasmid DNA was no longer detectable in tissues after 3 wk postadministration. RNA and protein expression was also found in the tissues and bloodstream. Animals previously primed by HSV infection and subsequently given IL-10 DNA via the nasal mucosa, showed diminished Ag-induced delayed type hypersensitivity reactions for up to 5 wk posttreatment. The mechanism of modulation involved diminished the Ag-specific proliferation and production of Th1 cytokines. The Ag-specific silencing effects persisted beyond the duration of detectable plasmid encoded protein and was maintained upon adoptive transfer of T cells into a plasmid-free environment. The silenced T cells were not a source of IL-10, and their anergic state was reversible by exposure to Ag in the presence of exogenous IL-2.

C hronic inflammatory reactions may be driven by sets of proinflammatory cytokines produced by stimulated T cells. This situation can be controlled by the application of inhibitory cytokines such as TGF-β and IL-10, which act in some way to silence the production or activity of the proinflammatory molecules (1, 2). Thus cytokine therapy represents a therapeutic option in clinical medicine, although administering molecules with short bioactivity as occurs with cytokine proteins is inconvenient and costly. The alternative approach of gene delivery is being explored although the relevant means of achieving cytokine gene expression requires definition. Recently, using a viral inflammatory lesion model, we compared the efficacy of IL-10 gene expression achieved by recombinant vectors or eukaryotic expression plasmid DNA (3). The latter approach provided the most durable efficacy and unlike viral vectors, was not subject to interference by the immune response. The naked plasmid DNA idea is under consideration for both vaccination and immunomodulation purposes, but the mechanisms of action and long-term effects of such an approach require further study (4, 5). Accordingly, long-term gene expression does occur especially at the i.m. injection site (6). These myocytes act as an enduring source of plasmid encoded protein, but it appears that the cells may not be the primary inducer of immune responses (7). Thus removal of the injected sites after inoculation has little effect on the level or nature of immunity (7). Indeed, it seems that dendritic cells transfected at the injection site and migrating subsequently to draining lymph nodes (DLN) are the principal source of immunogenic materials (7–12).

Most investigations on the fate of plasmid DNA and the origin of immunogenic material have focused on systemic administration, especially the i.m. route (13). Our laboratory has focused on mucosal delivery and has observed that this form of plasmid administration results in good immune induction as well as immune modulation (14–16). Following mucosal inoculation, protein expression was observed in the lung and DLN, but it is not clear how the plasmid disseminates to such sites or how mucosal plasmid DNA delivery results in the systemic immune induction and the immunomodulatory effects observed.

In the present report, we probe the fate of eukaryotic expression plasmid DNA after mucosal delivery and evaluate possible mechanisms by which plasmid DNA expressing IL-10 achieves immunomodulation. Our results indicate that plasmid DNA disseminates widely in the body following mucosal delivery. Distal tissues such as the dermis, bone marrow, and lymphoid tissues remote from the deposition site become seeded with plasmid DNA and, in addition, become accessible to cells presumably transfected at the inoculation site or draining lymphoid tissue. Whereas IL-10 protein can be demonstrated in the blood stream and can additionally be produced by transfected cells at local sites, the modulatory effects of IL-10 plasmid DNA appears mainly to result from central effects in T cell function. Thus T cells appear to be silenced for a period that extends beyond the duration of plasmid DNA gene expression. However, the silenced T cell reactivity was readily recovered by exogenous IL-2 treatment, indicating that the mechanism involved in long-term modulation is induction of an anergic-like state.

Materials and Methods

Animals

Five- to six-week-old female BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN) and 6- to 8-wk-old CB-17 SCID mice (Taconic Farms, Germantown, NY) were used for the study. BALB/c mice were housed conventionally, and SCID mice were housed in sterile microisolator cages.
in the animal facility. All food, water, bedding, and instruments were autoclaved or disinfected. All manipulations were performed in a laminar flow hood. To prevent bacterial superinfection, all SCID mice received treatment of sulfamethoxazole/trimethoprim (Biocraft, Elmond Park, NY) at the rate of 5 ml/200 ml of sterile drinking water. The investigators adhered to the guidelines set by the Committee on the Care of Laboratory Animal Resources, Commission of Life Sciences, National Research Council. The animal facilities of the University of Tennessee are fully accredited by the American Association of Laboratory Animal Care.

**Viruses**

HSV-1 KOS and HSV-1.17 strains were grown on a Vero cell monolayer (ATCC cat. no. CCL81), titrated, and stored in aliquots at −80°C until used.

**Plasmid DNA preparation**

Plasmid DNA encoding IL-10 under SV40 promoter was a gift of Dr. T. Mosmann (Edmonton, Alberta, Canada). A vector DNA without IL-10 gene was made by digestion of IL-10 plasmid DNA with EcoRI followed by ligation of the purified vector fragment. Plasmid DNA encoding β-galactosidase (pCMVβ, cat. no. 6177-1) and plasmid DNA encoding a red-shift variant of green fluorescent protein (pGFP, cat. no. 6084-1) were purchased from Clontech (Palo Alto, CA). Plasmid DNA was purified by polyethylene glycol precipitation as described previously (17). Protein expression of the different plasmids in eukaryotic cells (human embryonic kidney cells, HEK293) was determined as described elsewhere (15).

**Immunization with HSV**

BALB/c mice were anesthetized with methoxyflurane (Metophane; Pittman-Moore, Mundelein, IL) and received footpad injection of 10⁶ PFU of HSV-1 KOS.

**Plasmid DNA administration**

For intranasal (i.n.) administration, 3 wk postinfection, 200 g of plasmid DNA was suspended in 25 ml of sterile PBS and dropped onto the nasal mucosa. The mice injected the DNA as they breathed. Intramuscular administration was performed by injecting 100 μg of plasmid DNA in 50 μl of sterile PBS to each biceps muscles.

**Adoptive transfer protocol**

HSV-infected BALB/c mice received i.n. administration of 200 μg of IL-10 DNA or vector DNA. Around 4 wk later, the splenocytes were obtained from the mice and were enriched for T cells by passing through a nylon-wool column. These T cells were adoptively transferred i.v. to untreated SCID mice by tail injection. In some experiments, 500 U of rIL-2 (cat. no. 906011, Hemagen, Columbia, MD) was i.p. injected into SCID mice at days 0, 1, 2, and 3 following adoptive transfer. The enriched populations were analyzed by flow-cytometric analysis that usually consists of >85% of CD3+ T cells (>65% of CD4+ T cells and >25 of CD8+ T cells). The enriched cells were also analyzed for the presence of administered plasmid DNA by PCR that showed no amplified bands for β-gal or IL-10 plasmid DNA.

**Delayed type hypersensitivity (DTH)**

At different time points after IL-10 DNA treatment, test Ags in 30 μl of sterile PBS were injected into the ear pinnae of the HSV-infected BALB/c mice. The increase in ear thickness was measured 48 h later with a screw gauge meter (Oddest; H. C. Kroeplin, Schleuchtern, Germany) as described previously (18). Test Ags used were UV-inactivated HSV-1 KOS (10⁶ PFU before UV inactivation) or Vero cell extract. In a separate experiment, the ear of vector DNA-treated mice received the injection of 80 ng of IL-10 protein along with HSV Ag. At the same time or 3 h after HSV + IL-10 protein injection, the ear pinnae were injected with 1 μg of anti-IL-10 Ab (PharMingen, San Diego, CA; cat. no. 18140D) or 1 μg of rat IgG1 isotype Ab (PharMingen; cat. no. 20610D). The DTH response of the IL-10 DNA-treated mice was tested as described above. In some animals, at day 28 after i.n. treatment of IL-10 DNA, 1 μg of anti-IL-10 Ab or 1 μg of rat IgG1 isotype Ab were injected into ear pinnae along with HSV Ag. Similarly, SCID mice were tested for the DTH responses on the next day of adoptive transfer.

**Virus challenge**

On day 3 following adoptive transfer, the SCID mice were challenged with HSV as described elsewhere (19). Briefly, before challenge the left flank of mice was depilated by using a hair clipper (Oster Animal Hair Clipper, Milwaukee, WI) and a chemical depilator, Nair (Cater-Wallace, New York, NY). After depilation, the mice were anesthetized with methophane, and a total of seven scarifications were made on an approximate 5-mm² area using a 27-gauge needle. A total of 10 μl of 10⁶ PFU (100 LD₅₀) of HSV-1 strain 17 was added to the scarifications and gently massaged. Animals were inspected daily for the development of zosteriform ipsilateral lesions, general behavior changes, encephalitis and mortality. The severity of the lesions was scored as follows: 1 = vesicle formation; 2 = local erosion and ulceration; 3 = mild to moderate ulceration; 4 = severe ulceration, hind limb paralysis, and encephalitis; and 5 = death.

**HSV-specific lymphoproliferation**

This assay has been described in detail elsewhere (19). Briefly, at days 28 or 65 after plasmid DNA treatment, the splenocytes from vector or IL-10 DNA-treated mice were enriched for T cells by a nylon-wool column and used as responder populations. These T cells were restimulated in vitro with irradiated syngeneic splenocytes infected with UV-inactivated HSV (multiplicity of infection (moi) of 1.5 before UV inactivation) or irradiated naive splenocytes, and incubated for 5 days at 37°C. In some experiments, rIL-2 (10 U/well) was added. Con A (5 μg/ml) was used as a polyclonal positive control and incubated for 3 days. Eighteen hours before harvest, [³H]thymidine was added to the cultures. In at least five independent experiments, proliferative responses were tested in quadruplicate wells and the results expressed as mean cpn ± SD.

**IL-2 assay by ELISA**

On day 28 after IL-10 DNA treatment, splenocytes from the IL-10 DNA or vector DNA-treated BALB/c mice were obtained. The enriched T cells (5 × 10⁶ cell/ml) were restimulated in vitro with 5 × 10⁴ of irradiated syngeneic splenocytes infected with HSV-1 IgGOS or HSV-1 KOS splenocytes for 3 days at 37°C. Similar number of cells were stimulated with 5 μg of Con A as a polyclonal positive stimulator for 48 h. The culture supernatants were screened for the presence of IL-2 by ELISA assay. ELISA plates were coated with anti-IL-2 Ab (PharMingen, cat. no. 18161D) and incubated overnight at 4°C. The plates were washed three times with PBS containing 0.5% Tween-20 and blocked with 3% of nonfat dry milk for 2 h at room temperature. After washing, serially diluted samples and standard were added to the plates in duplicate, and incubated overnight at 4°C. After being washed, biotinylated anti-IL-2 Ab (PharMingen; cat. no. 18000D) was added and incubated 2 h at 37°C. The plates were washed and peroxidase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA; cat. no. 016-030-084) was added. The color was developed by adding the substrate solution (11 mg of 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid in 25 ml of 0.1 M citric acid, 25 ml of 0.1 M sodium acetate, 25 ml of 0.05 M hydrogen peroxide). IL-2 concentration was calculated with an automated ELISA reader (SpectraMAX 340, Molecular Devices, Sunnyvale, CA).

**Enzyme-linked immunospot (ELISPOT) for cytokine producing cells**

The detailed methods of ELISPOT assay for the quantification of cytokine producing cells were described previously (14). Twenty-eight days following IL-10 gene transfer, the splenocytes from vector or IL-10 DNA-treated mice were enriched for T cells by a nylon-wool column. The T cells were analyzed for IL-4 and IFN-γ spot-forming cells by ELISPOT. To generate cytokines, the T cells were stimulated in vitro with enriched dendritic cell populations obtained by the method of Nair et al. (20). The enriched dendritic cells were pulsed with UV-inactivated HSV (moi of 5 before UV inactivation) for the HSV-specific cytokine production. The T cells and stimulator DC (naive or pulsed) were added at a responder-to-stimulator ratios of 10:1, 5:1, 2.5:1, and 1.25:1 in 200 μl of RPMI 1640 medium with 10% fetal bovine serum per well into ELISPOT plates which were coated with anti-mouse IL-4 (PharMingen; cat. no. 18191D) or anti-mouse IFN-γ (PharMingen; cat. no. 18181D). After 9 h incubation, the plates were washed and biotinylated anti-mouse IL-4 (PharMingen; cat. no. 18042D) or biotinylated anti-mouse IFN-γ (PharMingen; cat. no. 18112D) were added. After 1 h of incubation at 37°C, 100 μl of alkaline phosphatase-conjugated streptavidin in PBS (1 μg/ml) was added and the plates were incubated for another 1 h. The spots were developed by using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as a substrate and color developed 24 h later under a dissecting microscope. Mean cytokine spot forming cells (SFC) ± SD from five independent experiment was expressed as results. In some experiments, rIL-2 (10 U) was added to culture wells.
IL-10 detection in serum after IL-10 gene transfer

Groups of mice received i.m. or i.n. treatment of either 200 μg of IL-10 plasmid DNA or vector. At different days, serum samples were collected from the mice and analyzed for the presence of IL-10 in the serum by ELISA assay. ELISA plates (DyNatech, Chantilly, VA) were coated overnight at 4°C with 2 μg of rat anti-mouse IL-10 (PharMingen; cat. no. 181141D). The wells in the plates were washed with PBS containing 0.05% Tween (PBS-T) and blocked with 3% milk for 2 h at 37°C. After washing, the sera samples and the rIL-10 (PharMingen; cat. no. 19281V) at a concentration of 1 ng/ml were added and serially diluted. The standard and samples were incubated overnight at 4°C. After washing with PBS-T, 1 μg of biotinylated anti-IL-10 Ab (PharMingen; cat. no. 18152D) per ml was added and incubated at 37°C for 2 h. After the wells were washed, peroxidase-conjugated streptavidin (Jackson ImmunoResearch) was added and incubated at 37°C for 2 h. Substrate was added for color development. IL-10 concentration was measured by automated ELISA reader (Spectra MAX 340; Molecular Devices). Each time point contains sera from at least four mice.

Isolation of DNA and RNA

At different time points following administration of pCMVβ, pGFP, or vector DNA (data not shown), several tissues including muscle, DLN, spleen, lung, liver, bone marrow, and ear were obtained. The tissue samples were minced into small pieces (or sometimes single cell suspensions of DLN and spleen were made). The preparations were treated with TRI Reagent (Research Center, Cincinnati, OH). Total RNA and DNA were isolated as described above. Briefly, RNA was extracted with chloroform and precipitated with isopropanol alcohol. The contaminating plasmid DNA was removed with RQ-1 RNase-free DNase (Promega, Madison, WI). The absence of plasmid DNA in the RNA preparations was verified by performing PCR on the total cellular RNA without reverse transcription. The RNA was isolated by manufacturer’s protocol. Briefly, RNA was extracted with chloroform and precipitated with isopropanol alcohol. The contaminating plasmid DNA was removed with RQ-1 RNase-free DNase (Promega, Madison, WI). The absence of plasmid DNA in the RNA preparations was verified by performing PCR on the total cellular RNA without reverse transcription.

RT-PCR

Total cellular RNA (10 μg/ml) was reverse transcribed using oligo(dT) primers and reverse transcriptase (Promega) according to protocol previously published (21). The contaminating plasmid DNA was removed by treatment with RQ1-RNase-free DNase and verified by PCR using both CMV-1/β-gal-2 (or GFP-1) primers and β-gal-1/β-gal-2 (or GFP-1/GFP-2) primers. The cDNA was made by the reverse transcription reaction incubated at 42°C for 90 min. The cDNA (5 μl) was subject to 35 cycles of amplification as described (22) using CMV-1 (sense) and β-gal-2 (or GFP-1 and GFP-2) and β-actin-1 and β-actin-2 primers. DNA isolated from the plasma was also amplified by 35 cycles of amplification using CMV-1 and β-gal-2 (or GFP-2) primers. The primers used followed: CMV-1, 5′-CCATTGAGCTCTGGAGAG-3′; β-gal-1, 5′-CTTTGACCAAAAGAAGCCTG-3′; β-gal-2, 5′-AAATGTGACGATACACG-3′; GFP-1, 5′-TCTGGAACCCCGACTAC-3′; GFP-2, 5′-CTCTGACGACCCAGCCTCC-3′; β-actin-1, 5′-GTCGGGCGCCCAGGCAACC-3′; β-actin-2, 5′-GGCTTATGTGACCCAGCAT-3′.

β-gal staining

At different days after pCMVβ DNA administration, DLN, spleen, lung, and ear tissues were isolated from the mice and fixed in 4% paraformaldehyde in PBS for 1 h at 4°C. The tissues were washed and stained for β-gal activity as described previously (22). Briefly, the tissues were incubated overnight with the substrate solution containing 2 mg/ml of 5-bromo-4-chloro-3-indolyl β-D-galactosidase, 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM MgCl₂, and 120 μl of 10% Nonidet P-40 and 100 μl of 1% sodium deoxycholate in 20 ml of PBS. After the reaction, the tissues were washed with PBS and fixed with 4% formaldehyde. The tissues were embedded in paraffin, sectioned at 5 μm, and counterstained with nuclear fast red.

FACS analysis

At day 10 following i.n. pGFP administration, PBC were isolated from anticoagulated blood. Following lysis of RBC, the PBC were incubated in the culture flask for 90 min at 37°C. The adherent cell populations were carefully separated from the nonadherent cells. After washing with DMEM containing 10% FBS, the cells (2 × 10⁶ cells/ml) were immediately fixed with 4% paraformaldehyde. The green fluorescence signals were analyzed by flow cytometry analysis using FL-1 channel. As a positive control, HEK293 cells were transfected with pGFP, and 60 h later the harvested HEK293 cells were fixed. The fluorescent signals from the cells were detected as described above.

Statistical analysis

Significant differences between groups were evaluated using the Student’s t test.

Results

IL-10 plasmid DNA suppresses Ag-specific immune responses in primed animals

Mice were infected systemically with HSV and 3 wk later received via the i.n. or i.m. routes 200 μg of IL-10 plasmid DNA either encoding IL-10 or vector DNA. At various days after this exposure, mice were tested for their cutaneous DTH responses to HSV Ag injected into the ear pinnae. Suppressed responses in IL-10 DNA-treated mice first became evident around 5 days posttreatment (Table I). However, the duration of suppression differed from i.m. to i.n. treatment. Such suppression by i.m. injection of IL-10 DNA lasted for 7 wk as reported earlier (3), whereas suppression had declined by day 45 in i.n. recipients of IL-10 DNA (Table I). Thus DNA encoding IL-10 administered mucosally as well as systemically is capable of suppressing Ag-induced inflammatory responses even when administered after sensitization has occurred. Sample mice in the group that showed suppressed DTH reactions were sacrificed 28 days post-IL-10 DNA administration to
was performed using both CMV-1/bKOS (1.5 moi before inactivation) or irradiated naive splenocytes, and incubated for 5 days. [3 H]-Thymidine was added to each well 18 h before harvest. The results show mean cpm ± SD for five independent experiments.

Proliferation index was calculated by cpm of HSV stimulation/cpm of unstimulation.

For the measurement of IL-2, the T cells were restimulated in vitro with irradiated syngeneic naive splenocytes or irradiated splenocytes infected with UV-inactivated HSV-1 KOS (1.5 moi before inactivation). Seventy-two hours later, culture sups were collected and analyzed for IL-2 by ELISA. The data represent mean concentration ± SD for five independent experiments. Con A (5 μg/ml) was used as polyclonal stimulator for proliferation and IL-2 production.

To analyze the number of IFN-γ and IL-4 producing cells, ELISPOT assay was performed. The T cells were restimulated in vitro with an irradiated enriched dendritic cell (DC) population infected with HSV (5 moi before UV inactivation) and incubated for 4 days. The number of cytokine SFC after naive DC stimulation was subtracted from the values of UV inactivated HSV-infected DC stimulation. The results represent mean SFC ± SD for five independent experiments.

Mechanism of modulation

The observations above indicate that mucosal delivery of IL-10 DNA results in systemic suppression of T cell reactivity. Several possibilities were considered to explain how mucosally delivered plasmid could result in such suppression. These included the passage of plasmid DNA to remote sites followed by cell transfection and expression at such sites. The plasmid DNA could be transported within cells that had been transfected at the mucosal delivery site or DLN. Alternatively, the IL-10 protein could be expressed locally and enter the blood stream to distribute to distant sites and actively suppress inflammatory responses. Further explanations could be that T cells were silenced in the local or distal lymphoid tissues by exposure to the IL-10 protein or that regulatory cells were induced that inhibited the responses of the Ag stimulated immune T cells.

Detection of plasmid DNA and transfected cells in blood and peripheral sites

To investigate whether plasmid DNA and the expression of encoded protein was present at the remote sites or in the blood stream, plasmids encoding β-gal or GFP were used to avoid the complication of detecting endogenous cytokine gene expression. Animals were given the plasmid DNAs or vector control mucosally and then killed at intervals and multiple tissues, including the separated plasma and PBC, were collected to detect DNA, RNA,

Table II. IL-10 DNA treatment suppresses HSV-specific T cell immune responses of primed animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Proliferation (cpm)b</th>
<th>IL-2 Production (pg/ml)c</th>
<th>SFC/5 × 10^6 Cellsd</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.n. IL-10 DNA</td>
<td>29,104 ± 2,148.3</td>
<td>7,432.1 ± 2,310.14</td>
<td>&lt;40</td>
</tr>
<tr>
<td>i.n. vector DNA</td>
<td>27,283 ± 3,174.5</td>
<td>18,390.8 ± 3,839.1</td>
<td>&lt;40</td>
</tr>
<tr>
<td>i.m. IL-10 DNA</td>
<td>28,284 ± 3,998.3</td>
<td>5,963.4 ± 1,438.94</td>
<td>&lt;40</td>
</tr>
<tr>
<td>i.m. vector DNA</td>
<td>30,148 ± 2,749.6</td>
<td>21,983.1 ± 4,145.3</td>
<td>&lt;40</td>
</tr>
</tbody>
</table>

*Groups of HSV immune mice received i.n. or i.m. administration of 200 μg of IL-10 DNA or vector DNA. At day 25 after IL-10 DNA treatment, spleen cells were obtained and enriched for T cells using a nylon-wool column.

†These responder T cells were mixed with irradiated syngeneic naive splenocytes (unstimulation) or irradiated splenocytes infected with UV-inactivated HSV-1 KOS, and incubated for 5 days. [3 H]-Thymidine was added to each well 18 h before harvest. The results show mean cpm ± SD for five independent experiments.

Distribution of plasmid DNA encoding β-gal and RNA expression in tissues following mucosal administration

Table III. Distribution of plasmid DNA encoding β-gal and RNA expression in tissues following mucosal administration

<table>
<thead>
<tr>
<th>Tissues</th>
<th>DNA</th>
<th>RNA</th>
<th>DNA</th>
<th>RNA</th>
<th>DNA</th>
<th>RNA</th>
<th>DNA</th>
<th>RNA</th>
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<tr>
<td></td>
<td>5 h</td>
<td>10 h</td>
<td>Day 1</td>
<td>Day 3</td>
<td>Day 7</td>
<td>Day 14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
<td></td>
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<tr>
<td>Blood (Ad.) cells</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td></td>
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<tr>
<td>DLN</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td></td>
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<tr>
<td>Spleen</td>
<td>++</td>
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<td>++</td>
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<td>++</td>
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<tr>
<td>Liver</td>
<td>++</td>
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<td>++</td>
<td>++</td>
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<td></td>
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<tr>
<td>Bone marrow</td>
<td>++</td>
<td>++</td>
<td>++</td>
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<td>++</td>
<td>++</td>
<td></td>
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<td>Ear</td>
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<tr>
<td>Lung</td>
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</table>

*Groups of mice received 200 μg of β-gal DNA, pGFP (not shown), or vector via nasal mucosa. At different time points, various tissues were obtained from some animals, put into TRI Reagent, and immediately frozen at −70°C. Anticoagulated blood was also collected and separated into plasma and PBC. Phenol/chloroform extraction was performed to isolate DNA from the plasma. Isolated PBC were washed with DMEM containing 10% FBS and incubated in the culture flask for 90 min at 37°C to separate adherent and nonadherent cells. After incubation, nonadherent cells were carefully obtained and adherent (Ad.) cells were harvested. The two cell populations were put into TRI Reagent and immediately frozen at −70°C. DNA and total RNA were isolated from the tissues per manufacturer’s protocol and analyzed for the presence of DNA by PCR analysis using CMV-1 and β-gal-2 primers (or CMV-1 and GFP-2 primers). To detect RNA expression, the isolated total RNA was treated with DNase, and PCR without reverse transcription was performed using both CMV-1/β-gal-2 primers and β-gal-1/β-gal-2 primers to exclude the presence of DNA. The RNA samples were reverse transcribed and amplified with β-gal-1 and β-gal-2 (or GFP-1 and GFP-2 primers). The intensity of amplified band was analyzed by the comparison of the band intensity of β-gal (or GFP) to β-actin. The experiment was performed four times independently. No amplified bands for β-gal (or GFP) DNA and RNA were detected in the samples of vector DNA-treated mice. Similarly, the multiple tissues were analyzed following i.m. injection of plasmid DNA. The data showed similar results except the plasmid DNA was present in plasma at 5 min later. The DNA in muscle tissues after i.m. injection was found at the end of experiment (day 49), whereas RNA expression was occasionally detected at day 28. In separate experiments, multiple tissues were examined for the presence of plasmid DNA following IL-10 DNA administration. The results showed similar pattern as β-gal experiment. +, positive; −, negative; +/−, occasionally positive.
or protein. The results recorded in Table III indicate that plasmid DNA received i.n. was present in blood by 5 h as detected by PCR using primer sets that can amplify only the introduced gene and its promoter (Fig. 1, Table III). This DNA persisted for at least 3 days in plasma and was evident in all tissues analyzed at days 1 and 7. These tissues included the ear pinna and spleen and in the latter plasmid DNA was detectable until at least day 14. Furthermore, in a separate experiment, the IL-10 plasmid DNA also disappeared from most tissues by day 20. The presence of DNA in the plasma means that early on plasmid DNA can transport via blood as cell-free plasmid DNA to multiple tissues, but because the technique used did not permit quantification whether or not the plasma route is more important than the intracellular route could not be established. Thus plasmid DNA was also present in separated and washed leukocytes.

Furthermore, semi quantitative RT-PCR revealed that GFP (data not shown) and β-gal RNA were present in separated blood cells (Fig. 1). The RNA was detectable from day 1 to day 7 and some RNA was still present at day 14. Even though RNA expression was not detected, the β-gal and IL-10 DNA could be still found in nasal tissues at day 21, but had disappeared by day 28 (data not shown). Regarding protein expression, 230–580 cells per 10⁵ adherent blood cells were positive for the GFP signal at day 10 but by 21 days protein expressing cells in the blood were undetectable (see Fig. 2). These data indicate that plasmid DNA transfection likely occurs at the sites of inoculation and that some cells enter the blood stream and circulate for prolonged but not indefinite periods. In addition, PBC might take up cell-free plasmid DNA within circulation.

RNA expression was also found at multiple peripheral sites (Fig. 1, Table III). In addition, protein-expressing cells were evident in lungs, DLN and spleen. Furthermore, some cells were also demonstrable at cutaneous sites (Fig. 2). Regarding the latter, a few protein-expressing cells could be also demonstrated in the inflammatory reactions induced at 7 days post-plasmid DNA inoculation but not in reactions induced at day 21 or beyond. Such data may indicate that the source of plasmid expression may be recruited cells from the blood stream as well as cells transfected at cutaneous sites by plasmid DNA arriving soon after mucosal inoculation. Our data could not distinguish whether the presence of RNA and protein expression at remote sites results from direct transfection by plasmid DNA at such sites or the consequence of seeding by cells transfected by plasmid at inoculation sites. These issues are under investigation.

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FIGURE 1. The presence of β-gal DNA (pCMV/β) and RNA expression at the different tissues. Groups of BALB/c mice received i.n. administration of 200 μg of plasmid DNA encoding β-gal (pCMV/β), plasmid DNA encoding GFP (pGFP, not shown), or vector DNA (data not shown). At different time points, several tissues were isolated from the mice and treated with TRI Reagent. Anticoagulated blood samples were also obtained and separated into plasma and blood cells. The blood cell samples were further separated into adherent and nonadherent cells. These two populations were treated with TRI Reagent, and DNA was extracted from the tissues as described in Materials and Methods. Plasmid DNA was amplified with CMV-1 and β-gal-2 primers (or CMV-1 and GFP-2 primers). To detect RNA expression, plasmid DNA was removed by treatment with RQ-1 RNase-free DNase and the absence of plasmid DNA was verified by PCR analysis without reverse transcription. Total RNA was reverse transcribed and amplified with β-gal-1 and β-gal-2 (or GFP-1 and GFP-2 primers). The figures represent β-gal DNA (~870 bp) and RNA (~400 bp) expression. Multiple samples from vector DNA received mice did not show positive signals for β-gal DNA and RNA expression. Similar results were obtained after i.n. administration of pGFP (data not shown). Lane 1, DLN; lane 2, spleen; lane 3, ear; lane 4, lung; lane 5, liver; lane 6, bone marrow; lane 7, adherent blood cells; lane 8, plasma.

FIGURE 2. β-Gal activity in the tissues after β-gal DNA (pCMV/β) administration. Groups of mice received pCMV/β, pGFP, or vector DNA (data not shown) via the nasal cavity. Seven days later ear tissues (A, magnification ×200; B, magnification ×400), lung (C, magnification ×100), DLN (D, magnification ×200), and spleen (E, magnification ×200) were obtained from the pCMV/β-treated mice, and stained for β-gal activity as described in Materials and Methods. Adherent blood cells (F, magnification ×600) were isolated from the vector DNA- (data not shown) or pGFP-treated mice and fixed with 4% paraformaldehyde. The cells expressing GFP were identified by fluorescence microscopy. Tissues or cells from vector DNA-treated mice were negative for β-gal activity and green fluorescence. Also, the number of cells expressing GFP was analyzed by flow cytometry at day 10 post-DNA treatment. The results follow: i.n. treatment of pGFP, 280–580 cells/10⁵ adherent blood cells; i.m. injection of pGFP, 350–790 cells/10⁵ adherent blood cells.
Local anti-IL-10 does not restore the suppressed DTH reaction by IL-10 DNA. Groups of HSV-immune mice received i.n. treatment of 200 μg of IL-10 DNA or vector. At 28 days post-DNA treatment, the vector-treated mice were tested for the DTH responses as described in Materials and Methods (upper panel). Some of the mice were injected into right ear pinnae with 20 μl of HSV-1 KOS and with 80 ng of IL-10 protein. At the same time or 3 h later 1 μg of isotype or anti-IL-10 Ab was injected into the ear pinnae. The IL-10 DNA-treated mice were injected with HSV-1 KOS or vero cell extract along with either 1 μg of isotype or anti-IL-10 Ab (lower panel). The increase in ear thickness was measured at 48 h later. Each group consists of at least seven mice. Similar results were obtained from the IL-10 DNA-treated mice tested at day 10 post-DNA treatment. Statistically significant between IL-10 protein-treated and either PBS or IL-10 protein plus anti-IL-10 DNA-received group, p < 0.05.

Active suppression is not involved in long-term modulation of inflammatory response

The initial mechanism considered was that suppression resulted from the effects of IL-10 protein produced at the sites of plasmid DNA transfection, and this protein actively inhibited the response at the tissue site of inflammation. For this mechanism to occur, IL-10 protein should be present in the bloodstream and tissues. To show that IL-10 protein could inhibit DTH, purified IL-10 was injected into the ear pinnae of HSV-primed mice at the same time as Ag. A single injection of IL-10 protein to the local inflammatory site was sufficient to mediate inhibition as observed previously by others (23). Furthermore, the subsequent injection (3 h later) of anti-IL-10 abrogated the inhibitory effect of the IL-10 protein (Fig. 3). Abrogation also occurred if the anti-IL-10 was injected at the same time as IL-10 protein and Ag. Following mucosal administration of IL-10 plasmid DNA, IL-10 protein was evident in serum in minimal amounts in some animals by day 2 and peaked around 8 days postinoculation (Fig. 4). Most animals still had measurable IL-10 on day 14, but by day 20 IL-10 protein was undetectable. Therefore, the increase of serum IL-10 level might explain the inhibition of DTH responses. However, the inhibitory effect of IL-10 DNA administration persisted for at least 2–3 wk beyond the time of detection of serum IL-10. Furthermore, in other experiments, anti-IL-10 Ab was injected along with Ag into the ears of HSV-primed mice that 10 or 28 days previously were given IL-10 DNA i.n. The anti-IL-10 treatment failed to affect the extent of the DTH reaction even though anti-IL-10 could reverse the inhibitory effects by rIL-10 treatment (Fig. 3). Furthermore, only very few cells expressing proteins could be found at inflammatory sites at day 14, and the effects of plasmid DNA modulation persisted beyond the time of plasmid DNA, RNA, or protein demonstration at peripheral sites (or even the central lymphoid tissue). Such observations likely mean that the long-term inhibitory effect on the cutaneous inflammatory reaction was not the consequence of IL-10 protein expression at the local site. This observation argues against active suppression as an explanation for long-term modulation by IL-10 DNA.

T cell silencing and regulatory cells

An alternative mechanism of modulation by IL-10 DNA could involve silencing of Ag-reactive T lymphocytes or the induction of regulatory cells in the DLN or spleen by exposure to IL-10 protein. The regulatory effect could result from the action of other sets of T cells (such as Tr1 cells described by others (24)). A series of experiments were designed to evaluate these issues. First, it was supposed that if T cells were silenced as a consequence of exposure to IL-10, their inhibitory activity should be maintained upon transfer to a neutral environment lacking IL-10 protein. This issue was evaluated by adoptively transferring enriched T cells from HSV primed and IL-10 DNA-treated mice (4 wk post-IL-10 DNA treatment) into recipient SCID mice. The transferred cell population lacked adherent cells and was taken at a time when protein and RNA expressing cells were no longer present as detected in parallel experiments in mice given β-gal marker plasmid DNA. Furthermore, IL-10 DNA could not be demonstrated by PCR in the adoptively transferred cell populations (data not shown). Thus apparently plasmid DNA free lymphocytes were transferred into the SCID mice.

One day after treatment, the SCID mice were tested if the adoptive transfers could support a DTH response. SCID mice, which received transfers from HSV-primed mice treated with vector DNA, served as positive controls for DTH reactions. As shown in Table IV, the recipients of T cells from IL-10 DNA-treated mice showed inhibited inflammatory responses. After testing animals for DTH reactions, the groups of SCID mice used for adoptive transfer were challenged on the scratched skin with HSV virus.
Animals were followed for the development of cutaneous zosteriform lesions as described previously (19). As shown in Table IV, whereas SCID mice that received HSV immune lymphocytes from vector-treated mice were protected from zosteriform lesions and died. Some level of immunity was still evident, however, because animals did survive longer than control SCID mice that received no adoptive transfers. The above observations provide evidence of silencing of effector cells or the presence of inhibitory cells in the transfer population. Thus in some systems T cells exposed to IL-10 protein can become inhibitory and also become themselves a source of IL-10 (24). Such cells have been termed Tr1 cells (24).

To analyze for the presence of regulatory cells, splenocytes were taken from the HSV-infected mice at day 28 following i.n. administration of IL-10 DNA. Such splenocytes, enriched for T cells by fractionation on nylon-wool columns, were mixed at a 1:1 ratio with nylon-wool nonadherent cells from HSV-primed mice that were not subsequently exposed to modulatory IL-10 plasmid DNA. The cell mixtures were transferred into syngeneic SCID mice and the animals tested for DTH responses 1 day later. No evidence for inhibition by the IL-10 exposed population was observed (data not shown). Furthermore, no evidence for an increase in the number of IL-10-producing cells was observed by intracellular IL-10 cytokine staining in the enriched cell populations taken at day 28 post-IL-10 DNA treatment (data not shown). These observations provide no support for the operation of an inhibitory cell population such as Tr1 cells.

### Table IV. Transfer of suppressed T cell immunity by IL-10 DNA to SCID

<table>
<thead>
<tr>
<th>Adoptive transfer</th>
<th>DTH Responses, Mean Increase (×0.01 mm)</th>
<th>Zosteriform Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1 KOS</td>
<td>Vero cell extract</td>
</tr>
<tr>
<td>Untransferred</td>
<td>1.2 ± 0.4</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>T cells from vector-treated mice</td>
<td>13.1 ± 0.6</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>T cells from i.n. IL-10 DNA- treated mice</td>
<td>7.3 ± 1.5</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

* Groups of HSV-infected BALB/c mice were given 200 µg of IL-10 DNA or vector DNA via nasal mucosa. At day 28 after IL-10 DNA administration, 10⁷ cells of an enriched T cell population from the mice was adoptively transferred into SCID mice via tail vein. The next day, DTH reaction was elicited in the SCID mice and measured 48 hours later. The results show mean increase of ear thickness ± SD. Challenge for the development of zosteriform lesions was performed by infecting 10⁶ PFU of HSV into the scratched skin of the SCID mice at day 3 following adoptive transfer. At day 20 postchallenge, the number of mice that showed lesions and average scores were determined. The presence of IL-10 DNA in the enriched population was tested by PCR, but no amplified signals were demonstrated. To analyze the number of IL-10-producing cells, intracellular cytokine staining for IL-10 was performed in the enriched cells from IL-10 DNA or vector treated mice, but no significant change in the number of IL-10-producing cells was identified (IL-10-positive cells in enriched population from vector-treated mice; <0.4%; IL-10 positive cells in enriched population from IL-10 DNA treated mice; <0.5%).

rIL-2 treatment reverses silenced T cell reactivity

Some reports have indicated that inhibitory effects of IL-10 on T cell functions is reversible by antigenic stimulation in the presence of exogenous IL-2 (25). To evaluate whether the T cell silence was affected by IL-2, in vitro and in vivo experiments were performed on T cells collected 4 wk after mucosal administration of IL-10 DNA. As shown in Table V, in vitro stimulation of silenced T cells with Ag in the presence of exogenous rIL-2 supported both proliferation and IFN-γ SFC responses.

To measure the effects of rIL-2 on T cell silencing in vivo, SCID mice were given rIL-2 at same time as adoptive transfer of T cells from IL-10 DNA-treated mice. Recipient mice were given additional IL-2 injection on days 1, 2, and 3. The animals were tested both for the magnitude of HSV-specific DTH reactions as well as their resistance to systemic challenge by HSV virus. The results shown in Table VI indicates that the IL-2 treatment reversed the silencing effects in that animals both developed DTH reaction and showed resistance levels to HSV comparable to that mediated by immune T cells from vector-treated animals. Our data indicate that the T cell silencing effect mediated by IL-10 appears reversible by antigenic stimulation in the presence of exogenous IL-2.

### Discussion

This report shows that mucosal administration of plasmid DNA leads to rapid and widespread plasmid DNA distribution around the body. The inoculation route represents a useful method of

### Table V. rIL-2 restores the suppressed T cell reactivity by IL-10 DNA

<table>
<thead>
<tr>
<th>Responder T cells</th>
<th>Proliferation (cpm)</th>
<th>IL-2 Production (pg/ml)</th>
<th>SFC/5 × 10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Con A stimulation</td>
<td>HSV stimulation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Con A stimulation</td>
<td>HSV stimulation</td>
<td>Unstimulated</td>
</tr>
<tr>
<td></td>
<td>IFN-γ</td>
<td>IL-4</td>
<td></td>
</tr>
<tr>
<td>T cells from vector-treated mice</td>
<td>32,181 ± 3,833.1</td>
<td>22,181.4 ± 3,761.5</td>
<td>31.7</td>
</tr>
<tr>
<td>Day 28 T cells from i.n. IL-10 DNA-treated mice</td>
<td>30,169 ± 2,723.4</td>
<td>3,522.8 ± 258.3³</td>
<td>7.5</td>
</tr>
<tr>
<td>Day 28 T cells from i.n. IL-10 DNA-treated mice</td>
<td>IL-2</td>
<td>ND</td>
<td>3,269.4 ± 3,138.1⁴</td>
</tr>
<tr>
<td>Day 65 T cells from i.n. IL-10 DNA-treated mice</td>
<td>35,184 ± 2,026.7</td>
<td>20,925.2 ± 2,111.5</td>
<td>30.6</td>
</tr>
</tbody>
</table>

* Groups of HSV immune mice were given 200 µg of IL-10 DNA or vector DNA via i.n. administration. At days 28 and 65 after IL-10 DNA treatment, splenocytes were obtained and enriched for T cells using a nylon-wool column. HSV-specific proliferation and cytokine assay were performed as described earlier. rIL-2 was added at 10 U/well.

³ Statistically significant between vector and IL-10 DNA treated group, p < 0.05.

⁴ Statistically significant between rIL-2-treated and PBS-treated group, p < 0.05.
modulating immune responsiveness with cytokine DNA. Dissemination likely occurred via the blood stream because plasmid DNA was present in plasma from day 1 until day 3 after nasal administration. The plasmid DNA was detectable in all tissues analyzed including lungs, DLN, spleen, liver, bone marrow and even the noninflamed dermis of the ear pinnae. Except for the site of administration, plasmid DNA was no longer detectable in tissues after 3 wk postinoculation. RNA expression was also detected in the tissues. In addition to plasmid DNA, the bloodstream also contained protein-expressing cells and secreted protein. Accordingly, animals given plasmid encoding GFP had detectable protein expressing cells until at least day 10 following i.n. administration. Secreted protein was present between day 6 and 14 following i.n. inoculation of IL-10 DNA. Animals previously primed by HSV infection and subsequently given IL-10 DNA i.n. showed modulated Ag-induced cutaneous inflammatory reactions for up to 5 wk postadministration. The mechanism of modulation involved diminished Ag-induced proliferation and the production of Th1 cytokines such as IL-2 and IFN-γ. The modulatory effect likely proceeded by multiple mechanisms, but because suppressed Ag-specific T cell functions persisted beyond the duration of detectable plasmid encoded protein and was maintained upon adoptive transfer of T cells into a plasmid-free environment, one of the modulatory effects was assumed to involve silencing of Th2 cell reactivity. Such silenced T cells were not themselves transfected by plasmid DNA and not a source of IL-10. Moreover, their anergic state was reversible by exposure to Ag in the presence of exogenous IL-2.

The observation that plasmid DNA was evident in plasma following intranasal inoculation was surprising, although such has been reported after systemic injection (26–28). How plasmid DNA gains entrance from a mucosal surface into the blood stream was not resolved. Likely routes could be through the highly vascularized nasal cavity or lung alveoli (29). In fact, protein-expressing cells were readily detectable in the lung. Others have reported that the circulation half-life of plasmid DNA is brief and of the order of 5 min to 16 min (26, 27, 30, 31). However, in our studies plasmid DNA was detectable for at least 3 days after a single mucosal administration. This finding may imply that seeding occurs over a lengthy period or that some plasmid DNA may be protected from breakdown by binding to cells or by being bound to some serum protein. Some support for latter idea has been reported by others (27, 32, 33). The presence of free plasmid DNA in the plasma could mean that this is the primary means by which plasmid DNA disseminates widely to distal tissues.

Table VI. rIL-2 treatment restores the silenced T cell immunity by IL-10 DNA in vivo

<table>
<thead>
<tr>
<th>Adoptive Transfer</th>
<th>Treatment</th>
<th>DTH Responses, Mean Increase (×0.01 mm)</th>
<th>No. of mice with lesions/no. of mice used</th>
<th>Zosteriform Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>T cells from vector-treated mice</td>
<td>PBS</td>
<td>14.9 ± 1.2</td>
<td>0/6</td>
<td>0.2</td>
</tr>
<tr>
<td>T cells from i.n. IL-10 DNA-treated mice</td>
<td>PBS</td>
<td>6.4 ± 1.1</td>
<td>4/6</td>
<td>2.9</td>
</tr>
<tr>
<td>T cells from i.n. IL-10 DNA-treated mice</td>
<td>IL-2</td>
<td>12.8 ± 1.6</td>
<td>0/6</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* Groups of HSV-infected BALB/c mice received i.n. administration of 200 μg of IL-10 DNA or vector DNA. At day 28 after IL-10 DNA administration, 10^7 cells of an enriched T cell population from the mice was adoptively transferred into SCID mice via tail vein. DTH responses and HSV skin challenge were performed as described earlier. rIL-2 (500 U) and PBS were i.p. injected at day 0, 1, 2, and 3 following adoptive transfer. At day 20 postchallenge, the number of mice that showed lesions and average scores were determined. The presence of IL-10 DNA in the enriched population was tested by PCR, but no amplified signals were demonstrated. To analyze the number of IL-10-positive cells was identified.

* Statistically significant between SCID mice that received T cells from vector-treated mice and from IL-10 DNA-treated mice. p < 0.05.

* Statistically significant between rIL-2 and PBS-treated SCID mice which received T cells from IL-10 DNA-treated group.

An additional transport mechanism could involve transfected cells, which were detectable in the blood stream for a longer period than was free plasmid DNA. Thus by RT-PCR using RNA from washed leukocytes, signals were detectable for 2 wk, and by using the nonsecreted protein GFP, transfected cells were evident for at least 10 days. The nature of the transfected cells was not established but were likely mainly macrophages and dendritic cells. In other studies, macrophages were shown to rapidly take up plasmid DNA via their scavenger receptor following i.v. plasmid DNA administration (27, 34). A recent report also showed macrophages containing GFP were evident in blood following i.m. injection of plasmid DNA encoding GFP (35). Other reports have indicated that plasmid DNA transport, at least from the site of muscle or skin inoculation to the DLN, proceeds via transfected dendritic cells (8–12). It will be of interest to further identify the nature of transfected blood cells as well as define the localization of their transfection. Possibilities include the bloodstream itself, the inoculated nasal mucosa, or the DLN. We are currently attempting to verify the latter idea by comparing the number of transfected cells in the circulation of normal mice with LTα k/o animals that lack lymph nodes (36, 37). It seems likely, however, that not all cells were transfected at the local lymph node or blood stream because cells of nonmyeloid type were evident in peripheral tissues. Thus occasional cells with fibroblast morphology were evident in noninflamed skin sections in animals inoculated mucosally with β-gal plasmid DNA. These observations may mean that plasmid DNA may exit the blood stream and also transfetct cells at tissue sites. Experiments are underway to further investigate these issues.

Our studies demonstrate that mucosal delivery of plasmid DNA encoding IL-10 provided long-term suppression of an Ag-induced inflammatory reaction orchestrated by CD4+ Th1 T lymphocytes. Accordingly, mice infected with HSV and subsequently given IL-10 DNA had suppressed cutaneous DTH reactions to viral Ags. Suppression took a few days for onset but persisted for 5 wk. Many investigations have demonstrated that IL-10 can modulate Th1-mediated functions with multiple mechanisms likely occurring (2). These include inhibition of cytokine production and action on target cells, as well as inhibition of recruitment of nonspecific cells to inflammatory sites (2, 38). IL-10 protein in lesion sites can mediate such effects as was demonstrated in this communication as well as by others (23). However, whereas direct active suppressive effects of IL-10 protein in lesions might account for part of the suppression at least in the early phase, this mechanism could not provide the complete explanation for our observations. Thus, the suppression persisted well beyond the duration of detectable IL-10 protein...
in serum (6–14 days) or even protein-producing cells (up to 3 wk) at least as measured by detection of a marker protein. In addition, whereas the modulatory effect of exogenous IL-10 protein could be neutralized by anti-IL-10 injected into inflammatory sites, similar injections were without effect on suppression induced by mucosal plasmid DNA administration. Furthermore, the IL-10 DNA suppressed activity of T cells was retained when such cells were adoptively transferred to an environment lacking IL-10 plasmid DNA.

We interpret such observations to mean that some form of central suppression of lymphoid function had occurred. This notion was further supported by the observation that Ag-specific lymphocyte responses measured in vitro were suppressed especially as regards Th1 cytokine production. We have referred to the inhibited effect as T cell silencing and favor this mechanism over one of inhibition by a separate set of regulatory cells. Thus, in some systems investigators have demonstrated that long-term stimulation of CD4+ T cells in the presence of IL-10 leads to the production of a subset of T cell (Tr1 cells) which secret IL-10 and inhibit the action of Th1 cells (24). Using adoptive transfers of cell mixtures into recipient mice and measuring their DTH reactions, no evidence for regulatory effects was forthcoming. Furthermore, in T cell populations taken from suppressed mice, no evidence for an increase in the number of IL-10 producing cells was observed. Although we lacked positive evidence for the existence of regulatory Tr1-like cells after mucosal IL-10 DNA administration, this issue is being further investigated.

The nature of T cell silencing requires further study, but this is difficult to accomplish in the system we described because the viral Ag specific CD4+ T cell frequency is low. However, the silenced cells would appear to be in a reversible state of anergy, because in addition to recovering their former function with time, their Ag-specific function could be recalled by stimulating cells in the presence of exogenous IL-2. This IL-2 reversible effect was evident both in vitro as well when the suppressed cells were adoptively transferred to SCID mouse recipients given IL-2 and Ag. Interestingly, whereas the adoptive transfer of silenced cells to SCID mice failed to confer protection against HSV challenge, the effect was reversed in recipient of IL-2. In other system involving IL-10 protein mediated unresponsiveness of CD4+ T cells, the effect was reversible by culture of the cells in vitro with exogenous IL-2 (25). However, in another system unresponsiveness induced by IL-10 appears to be irreversible by IL-2 (39). It could be that the IL-10 treatment down-regulates IL-2 receptor α-chain on the T cells, inhibiting IL-2 signaling. In our system, IL-2 could functionally reverse the Ag unresponsiveness state, indicating that the signaling pathway through IL-2 receptor was not inhibited by IL-10 treatment. In fact, we could detect no change on the expression of IL-2 receptor α-chain on the T following IL-10 DNA administration (unpublished data).

In conclusion, mucosal delivery of plasmid DNA results in widespread distribution in the body. This distribution may occur by transport of free plasmid DNA as well as by transfected cells. However, persistence of the plasmid DNA is not indefinite and was far shorter than up to 9 mo reported by some to occur following i.m. plasmid DNA administration (40). Nevertheless, this shorter duration represents a potential advantage of mucosal delivery in some clinical situations. One such instance may be when using gene delivery to express immunomodulators. Thus modulating an unwanted inflammatory immune reaction may concomitantly render recipients more susceptible to infection by agents where defense depends on the function of the type of immune response being suppressed. In fact, in the case of HSV infection, the CD4+ Th1-mediated inflammatory reaction, while undesirable and tissue damaging in certain locations such as the eye (41), is actually the principal means of immune control following HSV infection (19, 41). Suppressing the response renders animals more susceptible to infection and virus can spread to critical tissues such as the brain (42). Indeed, in our studies using adoptive transfer experiments we showed that T cells from HSV primed IL-10 DNA-treated mice were less immunoprotective against HSV challenge than were T cells from untreated animals. Accordingly, modulating immunity using cytokine gene delivery somewhat resembles a Faustian dilemma!

References


