Modulation of Viral Immunoinflammatory Responses with Cytokine DNA Administered by Different Routes

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The efficacy of plasmid DNA encoding cytokine administered by different routes, systemic or surface exposure, was evaluated and compared for their modulating effects on subsequent lesions caused by infection with herpes simplex virus (HSV). Systemic or topical administration of both interleukin-4 (IL-4) and IL-10 DNA but not IL-2 DNA caused a long-lasting suppression of HSV-specific delayed-type hypersensitivity response. IL-4 or IL-10 DNA preadministration also modulated the expression of immunoinflammatory lesions associated with corneal infection of HSV. Suppression of ocular lesions required that the DNA be administered to the nasal mucosa or ocular surfaces and was not evident after intramuscular administration. The modulating effect of IL-10 DNA was most evident after topical ocular administration, whereas the effects of IL-4 DNA given by both routes appeared to be equal. Preexposure of IL-4 DNA, but not IL-10 DNA, resulted in a significant change in Th subset balance following HSV infection. Our results indicate that the modulating effect of IL-4 or IL-10 DNA may proceed by different mechanisms. Furthermore, our results suggest that surface administration of cytokine DNA is a convenient means of modulating immunoinflammatory lesions.

The realization that plasmid DNA eukaryotic expression vectors could be used to induce immunity against the encoded protein following systemic or even mucosal administration, opened up a novel means of vaccination (4, 10, 11, 14, 23). Many harbor the hope that DNA vaccines might replace some existing preparations and may even be successful against infectious agents which currently lack effective vaccines (15). The naked-DNA approach also holds promise as a convenient means of achieving gene transfer, since the vehicle contains no protein recognizable to the host and even the existence of specific antibody to the encoded protein appears not to block gene expression (16). Consequently, DNA vaccines represent a potential method of boosting or modulating the nature of immunity in previously primed animals.

Previous studies from this and other laboratories have shown that the plasmid DNA approach can be used to express natural molecules such as cytokines which can influence the nature of immune responses (2). The administration of DNA encoding a cytokine may affect the extent and type of immune reaction to coadministered antigens (1). Furthermore, recently it became evident that plasmid DNA encoding a cytokine such as interleukin-10 (IL-10) can influence the severity of immunoinflammatory lesions, even when administered during the disease process (2). In our previous study, in which DNA encoding IL-10 was shown to attenuate herpes simplex virus (HSV)-induced ocular immunoinflammatory lesions, it was necessary to administer the plasmid directly to the ocular tissue. Intramuscular (i.m.) administration was without beneficial effect (2). Such results indicated that the route of plasmid DNA exposure may critically influence efficacy.

In the present report, we have further investigated the influence of the administration route, using three cytokine-encoding DNAs for their ability to modulate the expression of both ocular and cutaneous inflammatory responses caused by HSV. Our results show that prophylactic treatment by either systemic or surface exposure with IL-4 or IL-10 DNA, but not IL-2 DNA, markedly suppressed cutaneous HSV-specific delayed-type hypersensitivity (DTH) reactions. Ocular lesions, in contrast, were inhibited by both IL-4 and IL-10 DNA pretreatment but only when given via the intranasal (i.n.) or ocular route and not when administered systemically. Since only IL-4 DNA but not IL-10 DNA preexposure resulted in a significant change in the subsequent Th1 and Th2 HSV-specific T-cell response, the inhibition observed was assumed to proceed by different mechanisms. Suppression caused by IL-10 DNA may depend on local cytokine expression at the inflammatory site itself, whereas the effect of IL-4 DNA may result mainly from central immune modulation. The implications of our observations regarding the use of cytokine DNA to modulate immunoinflammatory disease are discussed.

MATERIALS AND METHODS

Mice. Female BALB/c mice (H-2b), 3 to 4 weeks old, were purchased from Harlan Sprague Dawley (Indianapolis, Ind.) and acclimated for 1 week prior to experimentation. All experimental procedures were followed with Association of Research in Vision and Ophthalmology resolutions on the use and care of laboratory animals. The animal facility of the University of Tennessee is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Virus. HSV type 1 (HSV-1) strains RE and KOS were grown on Vero cells (CCL70; American Type Culture Collection, Rockville, Md.). The virus was maintained in Dulbecco modified Eagle medium (DMEM) containing 2% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Grand Island, N.Y.) and titrated by the standard protocol (22). Virus stocks were aliquoted and stored at −80°C.

Plasmid preparation. Plasmid DNA encoding murine IL-2 with the cytomegalovirus promoter was a gift from H. Ertl (PcDNAIII IL-2) (Philadelphia, Pa.). Plasmid DNA encoding murine IL-10 containing the simian virus 40 promoter was provided by T. Mosmann (Edmonton, Alberta, Canada). Plasmid DNA expressing murine IL-4 was generated in our laboratory, using IL-4 cDNA from American Type Culture Collection (catalog no. 37561). All plasmids were purified by polyethylene glycol precipitation by the method of Sambrook et al. (21), with some modifications as previously described (2). The expression of each plasmid DNA was identified by reverse transcription-PCR, enzyme-linked immunosorbent assay (ELISA) (for IL-10) or bioassay (for IL-2 and IL-4). PcDNAIII was used as a control vector.

Plasmid DNA administration. To administer DNA, mice were deeply anesthetized with methoxyflurane (Metophane; Pittman-Moore, Mundelein, Ill.). For i.m. administration, mice were injected into the tibialis or biceps muscles of both legs with 100 μg of plasmid DNA in 25 μl of Hanks balanced salt solution three
times at weekly intervals. i.n. immunization was performed three times at weekly intervals with 200 μg of plasmid DNA in 25 μl of Hanks balanced salt solution. For intracocular (i.o.) administration, corneas were slightly scarified with a 27-gauge needle, and 100 μg of plasmid DNA in 4 μl of Hanks balanced salt solution was applied to the corneas three times at weekly intervals.

**Corneal infection and clinical observation.** On the day after the last administration of DNA, mice were anesthetized and the scarified corneas were infected with 10^6 PFU of HSV-1 RE in 4 μl of sterile phosphate-buffered saline (PBS). The corneas and eyelids were gently massaged. The animals were examined daily after infection, and the severity of stromal keratitis was graded from 0 to 5 by slit lamp biomicroscopy (Keeler Instrument, Biomag, PH) as follows: 0, clear eye; 1, local or mild limbal neovascularization; 2, abundant neovascularization and mild corneal opacity; 3, opaque cornea and iris vessel engorgement; 4, severe corneal opacity, and iris not visible; 5, complete corneal rupture and necrotizing stromal keratitis.

**Cytokine detection in tissues.** Three days after i.m., i.n., or i.o. administration of plasmid DNA encoding either IL-2, IL-4, or IL-10 and control vector, the corneas and cervical lymph nodes (LN) were collected and transferred to DMEM with 10% FBS. Additionally, skeletal muscles and popliteal LN were also obtained from the i.m. treatment group. The samples were frozen at −80°C, thawed at 37°C, homogenized for 45 s (Pro 200; ProScientific, Monroe, Conn.), and centrifuged for 2 min at 10,000 × g at 4°C. The supernatants were analyzed for IL-2, IL-4, or IL-10 production by ELISA. The wells in the plates were coated with 2 μg of rat anti-mouse IL-2, IL-4, or IL-10 antibody (catalog no. 18161D, 18191D, or 8141D, respectively; Pharmingen) at 4°C overnight. The wells were blocked with 3% milk for 1 h at 37°C. The samples and recombimant IL-2 (rIL-2), rIL-4, or rIL-10 (catalog no. 1921T, 1923T, or 19281V, respectively; Pharmingen) at a concentration of 1 ng/ml were added and serially diluted. The standard and samples were incubated overnight at 4°C. After the wells were washed, 1 μg of biotinylated anti-IL-2, -IL-4, or -IL-10 antibody (catalog no. 18172D, 18042D, or 18152D, respectively; Pharmingen) 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 1 h. The ELISA was performed as described previously (15).

**HSV-specific lymphoproliferation assay.** To test whether HSV-specific T-cell responses were affected by plasmid DNAs encoding cytokines, the animals were sacrificed approximately 21 days following infection. Two spleens were pooled and used as the responder population. This method has been described in detail elsewhere (15). Briefly, these responders were restimulated in vitro with irradiated syngeneic lymph node cells obtained from the infected mouse. The responder cells at 10^6/ml were added to the irradiated syngeneic splenocytes infected with UV-inactivated HSV (multiplicity of infection [MOI] of 1.5 prior to UV inactivation) or irradiated naive splenocytes and incubated for 5 days at 37°C. Eighteen hours before harvesting, [3H]thymidine was added to all culture wells. Harvested cells were assayed for radioactivity, and results were expressed as mean counts per minute ± standard deviation for five replicates per sample.

**DTH.** Eighteen days after infection, test antigens in 20 μl of PBS were injected into the ear pinna of anesthetized mice and the ear thickness was measured 48 h postinjection with a screw gauge meter (Oditest; H. C. Kroepelin GHBB, Schleiden, Germany) as described elsewhere (9). Test antigens used were UV-inactivated HSV-1 KO (10^6 PFU prior to UV inactivation) and Vero cell extract in the right and left ears, respectively. The mean difference between the thickness of the right and left ear was calculated. In separate experiments, 20 μl of IL-2 (10 ng/ml) and HSV-1 KO (10^6 PFU prior to UV inactivation) were injected in the right ear and 20 μl of HSV-1 KO was injected in the left ear and left footpad. For the control, 20 μl of HSV-1 KO and Vero extract were injected in the right ear and right footpad and in the left ear and left footpad, respectively.

**Virus isolation and titration.** To collect ocular virus samples, eyes were swabbed at different time points after HSV infection and samples were resuspended in 500 μl of serum-free DMEM. The samples were stored at −80°C until tested. Individual samples (125 μl) were further diluted, and viral titers were obtained by using a plaque assay performed on Vero cells as described elsewhere (22).

**Antibody analysis.** Serum samples from each mouse were collected at day 21 postinfection (p.i.) and analyzed individually for HSV-specific antibody (immunoglobulin G [IgG]) in a standard quantitative ELISA described in detail elsewhere (10). Briefly, serum was tested for IgG2a, IgG1, and total IgG, using polyclonal anti-mouse IgG (Jackson Immunoresearch) at a concentration of 1 ng/ml were added and serially diluted. The standard and samples were incubated overnight at 4°C. After the wells were washed, 1 μg of biotinylated anti-IgG2a, -IgG1, or -IgG antibody (catalog no. 18172D, 18042D, or 18152D, respectively; Pharmingen) 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 1 h. The ELISA was performed as described previously (15).

**Cytokine expression.** To determine whether the three cytokine DNA constructs employed were expressed, two approaches were used. First, human embryonic kidney cells (293 cells) were transfected in vitro with cytokine DNA. After 3 days of culture, supernatants were harvested and tested, without dilution, for the presence of cytokines. All three cytokines (IL-2, IL-4, and IL-10) were detectable (IL-2 and IL-4 measured by bioassay and IL-10 measured by ELISA) (data not shown). More importantly, in vivo expression of cytokine proteins was measured 3 days following DNA administration by various routes. As is evident in Fig. 1, cytokine DNAs were expressed, but the route of administration markedly affected the outcome. In ocular tissue, all three cytokine proteins were undetectable in ocular tissue following i.n. or i.m. DNA administration. Cervical LN (a draining LN for both ocular and nasal tissue) extracts were positive for all three cytokine proteins following i.o. or i.m. administration, but proteins were undetectable in the cervical LN following i.m. injection. The latter, however, resulted in...
cytokine expression in muscle and popliteal LN (data not shown).

Effect of prophylactic cytokine DNA on the subsequent expression of HSV-induced immunoinflammation. Groups of mice were injected on three occasions by different routes with cytokine or vector control DNA, after which animals were infected ocularly with HSV. Animals were evaluated clinically at intervals for the development of herpetic ocular lesions, sampled periodically for viral secretion in tears as well as antibodies in serum. Tests for DTH reactions were also performed. Around 21 days p.i., most animals were sacrificed and their tissues collected for immunological evaluation. As is apparent in Fig. 2, preadministration of both IL-4 DNA and IL-10 DNA by either the ocular or i.n. route led to significant suppression in the severity of ocular disease. The level of IL-10-mediated suppression was greater following i.o. administration (60% of eyes had score less than 2) than after i.n. treatment (36%). Both routes appeared equally effective when IL-4 DNA was administered (i.o., 55%; i.n. 47%). In contrast, i.m. administration of either IL-4 or IL-10 DNA had no apparent effect on the severity of herpetic stromal keratitis (HSK) lesions. Similarly, IL-2 DNA given by any of three routes failed to reduce the severity of HSK but instead appeared to exacerbate severity. The lesion scores in vector-control-treated animals were approximately equal to those occurring in untreated mice (data not shown). The influence of cytokine DNA administration on the expression of subsequent HSV-specific cutaneous DTH reactions was also measured (Fig. 3a). As with HSK, suppression resulted from preadministration of either IL-4 or IL-10 DNA, but not IL-2 DNA. All three routes of DNA exposure proved efficacious in suppression of DTH reactions, and inhibitory effects appeared prolonged. Suppressed DTH reactions were still present at 7 and 8 weeks p.i., respectively (Fig. 3b). Interestingly, in a separate experiment in which IL-10 protein was injected along with antigen during the elicitation of DTH reaction in sensitized animals, suppressed responses were evident in the IL-10-injected ear but not in the other ear (Fig. 4). Furthermore, DTH responses in distal sites such as footpads from the IL-10-injected site were unaffected. In addition, i.m. injection of IL-10 protein by the same protocol had no effect on DTH responses (data not shown). Thus, although the IL-10 DNA had widespread suppressive effect, the effect of IL-10 protein was confined to the injection site.

Effect of cytokine DNA pretreatment on HSV-specific immune responses. Samples taken at intervals from mice revealed little effect of cytokine DNA pretreatment on the duration or level of ocular viral secretion following infection (data not shown). However, blood samples examined on day 21 p.i. revealed changes in the IgG isotype ratio in mice which received IL-4 DNA by each of the three routes of administration (Fig. 5). The isotype pattern was consistent with a shift toward the Th2 profile. Such a shift was not evident in recipients of IL-10 or IL-2 DNA. As for T-cell function measured in
in animals sacrificed around 21 days p.i., once again recipients of IL-4 DNA showed a shift in antigen-induced cytokine production toward the Th2 pattern (Fig. 6). In such mice, numbers of splenic cytokine-forming cells (SFC) producing IFN-γ were reduced and SFC producing IL-4, IL-5, and IL-10 increased (the latter between 10- and 20-fold). IL-10 DNA recipients did have diminished numbers of IFN-γ SFC, but there was no significant elevation of Th2 cytokine-producing SFC. However, both IL-4 DNA and IL-10 DNA inhibited HSV-specific lymphoproliferation (Fig. 7). Taken together, our results indicate that IL-4 DNA administration shifts the T-cell reactivity pattern toward a Th2 profile, while IL-10 DNA exposure induces the downregulation of HSV-induced Th1 response rather than a shift. This suggest that the suppressed inflammatory response which resulted from both IL-4 and IL-10 DNA administration may proceed by different mechanisms.

**DISCUSSION**

This report addresses the issue of whether virus-induced inflammatory responses can be modulated by the preadministration of naked plasmid DNAs (eukaryotic expression vectors) encoding cytokines. Our results show that cytokine DNAs encoding IL-10 and IL-4 administered topically to the cornea or nasal surfaces do modulate the severity of ocular and cutaneous lesions associated with HSV infection. Both of the HSV lesions are considered to represent immunoinflammatory responses resulting primarily from antigen recognition by CD4+ T cells of the type 1 cytokine-producing profile (7, 13, 19). Not unexpectedly, control experiments with DNA encoding IL-2 or IFN-γ (data not shown) failed to modulate the severity of lesion expression. Although ocular inflammatory lesions were modulated by topical administration of IL-10 or IL-4 DNA, the same preparations given i.m. had no inhibitory effects. In contrast, however, both of the cytokine DNAs did cause suppression of cutaneous DTH lesions following i.m. administration, and this suppression persisted for at least 7 weeks. It is notable that nonreplicating plasmid DNA can affect the immune responses for a prolonged period following herpesvirus infection.

The essential mission of the present research was to evaluate the route of cytokine DNA exposure for their modulatory effects, since most previous studies using plasmid DNA either for vaccination or modulatory effects used systemic administration (1, 20). We have shown that plasmid DNA encoding certain HSV proteins given mucosally or to the ocular surface induced immune responses against the encoded protein (3, 10). Others have also shown that cytokine DNA given in a liposome formulation to the nasal mucosa may modulate the subsequent expression of allergic disease (12). Other studies, however, have not simultaneously compared routes for modulatory effects or studied numerous cytokine DNAs in parallel. This study shows that cytokine DNAs given topically, especially to readily accessible sites such as the nasal mucosa, provide a
novel and convenient means of managing unwanted inflammatory lesions. Although our present report deals with prophylactic cytokine DNA administration, at least with IL-10 DNA, topical application to early immunoinflammatory ocular lesions can have beneficial effects (2).

Our observation that the effect of cytokine DNA on ocular and cutaneous reactions associated with HSV infection differed according to the route of cytokine DNA administration was unexpected, since both lesions were assumed to involve similar cellular mechanisms of expression. As mentioned earlier, both lesions are assumed to largely represent CD4 T-cell orchestrated events with type 1 cytokines principally involved. However, our data showed that both IL-10 and IL-4 DNAs diminished DTH reactions, regardless of the route of cytokine DNA administration. Moreover, suppression persisted for a surprisingly long time (at least 7 weeks). By way of contrast, ocular lesion modulation did not occur following i.m. DNA administration. Modulation of these lesions was most evident following topical application of cytokine DNA directly to the corneal surface. This was especially true for IL-10 DNA application. Modulation of these lesions was most evident following topical application of cytokine DNA directly to the corneal surface. This was especially true for IL-10 DNA application. As was evident from in vitro measures of immunity in cytokine DNA-treated animals, the results of IL-4 DNA administration was to affect the nature of the subsequent antigen-specific T-cell immune response. In fact, even though certain motifs of DNA (CpG sequence) may cause Th1 differentiation nonspecifically, there was a shift toward the Th2 pattern in IL-4 pretreated groups, which was reflected by results of both T-cell cytokine measurement and Ig isotype ratios. Thus, the effect of IL-10 may be largely dependent on local action at sites of inflammation. Indeed, modulation of ocular lesions by IL-10 DNA was most efficient on ocular lesions when administered topically to the eye itself. Furthermore, following ocular administration of IL-10 DNA, IL-10 protein expression could be directly demonstrated (Fig. 1). IL-10 is well-known to reduce the capacity of antigen presentation and inhibit the production of proinflammatory cytokines such as IFN-γ, IL-1, and tumor necrosis factor alpha (17). In fact, we have found that topical ocular IL-10 DNA administration led to reduced tumor necrosis factor alpha production in ocular tissue (unpublished data). Our results did show, however, that i.n. administration of IL-10 DNA had some modulatory effects on HSK expression. This could be due to some IL-10 protein gaining access to the eye following i.n. administration, although we could not formally demonstrate this fact. It is known, however, that lymphoid tissue draining the eye and nasal cavity includes some of the same nodes, and IL-10 protein expression was demonstrated in cervical LN following i.n. administration. If IL-10 DNA operates by causing local expression in actual lesions themselves, then the most difficult obser-
vation to explain was that IL-10 DNA given topically or systemically suppressed DTH reactions and this effect persisted for some weeks. Recently, it was reported that repeated IL-10 protein exposure induced a particular T-cell population (Tr1) which produces mainly IL-10 but not IL-4 (5). It may be possible that IL-10 DNA administration induces such a T-cell population and that such cells gain access to cutaneous sites during DTH reactions.

Alternatively, following administration, DNA may gain access to cutaneous sites and persist there for weeks. The traffic pattern which follows DNA administration to various sites has not been elucidated. Surprisingly, DNA can be observed at remote locations from the point of deposition as detected by PCR or protein expression of markers such as β-galactosidase (β-Gal). Using β-Gal DNA, we have also found signals (expression) in the DTH inflamed ears following systemic or topical administration (unpublished observation). Indeed, we have shown that surface exposure of β-Gal DNA can induce gene expression in the distal tissues, such as cervical LN and spleen (3). Therefore, locally expressed IL-10 might serve to suppress DTH responses. In support of this idea, we showed that IL-10 protein administration caused inhibition of the DTH responses in the protein-injected ear but not at the distal sites.

How the DNA is transported to cutaneous locations and whether the process can cause therapeutic effects to become magnified are intriguing topics currently under investigation in our laboratory. Whatever the mechanism involved, our results serve to demonstrate that plasmid DNAs encoding cytokines administered to readily accessible surface sites are a convenient means of modulating immunoinflammatory lesions.

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REFERENCES


