To the Graduate Council:

I am submitting herewith a dissertation written by Massoud Daheshia entitled "Pathogenesis and Modulation of Herpetic Stromal Ketatitis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Barry T. Rouse, Major Professor

We have read this dissertation
and recommend its acceptance:

Stephen J. Kennel

Robert N Moore

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

PATHOGENESIS AND MODULATION OF HERPETIC STROMAL KERATITIS

A

DISSERTATION PRESENTED

FOR THE

DOCTOR OF PHILOSOPHY DEGREE

THE UNIVERSITY OF TENNESSEE, KNOXVILLE

MASSOUD DAHESHIA

DECEMBER 1997

ACKNOWLEDGMENTS

I would like to thank the members of my dissertation committee; Drs. Barry T. Rouse, Leon Potgieter, Robert Moore, Steve Kennel, Joyce Merryman and Philip Boschler. I also wish to thank my colleagues, the Departments of Microbiology, Pathology and Comparative and Experimental Medicine, UTCVM and the NIH / National Eye Institute for financial support.

ABSTRACT

This Ph.D. thesis deals with the pathogenesis and modulation of Herpetic Stromal Keratitis, in the murine model, caused by HSV-1 - infection of the cornea.

In the first part of this dissertation, early events in the course of herpetic stromal keratitis are analyzed. The results of in situ hybridization revealed that there is an early induction of proinflammatory molecules following ocular infection by HSV-1 in the stromal cornea. This induction is mainly due to the influx of inflammatory cells. However, the cells infected by HSV-1 were localized exclusively in the corneal epithelium, leading to the hypothesis that there is a physical separation between those cells infected by the virus and those cells producing proinflammatory molecules.

In the second part, the potential efficacy of an expression vector encoding IL-10 was evaluated for its' ability to overcome ocular proinflammatory disease. The results show that this new approach can be applied successfully to assist in the recovery and protection of an animal from specific ocular inflammatory disease. This notion, that an established inflammatory response and disease outcome can be controlled in vivo, opens up new areas of investigation for similar pathologic diseases.

In the third part, the potential use of ocular tissues as a route for immunization was investigated. The data showed that following topical ocular immunization there was induction of mucosal and systemic protective immunity to HSV-1. The protection was accompanied by induction of both humoral and cellular immunity.

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LIST OF ABBREVIATIONS

AC	anterior chamber of eye
APC	antigen presenting cell
B7-1 / B7-2	costimulatory molecules of myoblasts
B-gal	Beta-galactosidase
CLN	cervical lymph nodes
CMI	cell mediated immunity
CTL	cytotoxic lymphocytes
cDNA	complimentary DNA
DC	dendritic cells
DLN	draining lymph node
DTH	delayed type hypersensitivity
EAE	experimental allergic encephalomyelitis
GATA-3	transcription factor for cytokine gene expression
GM-CSF	granulocyte-macrophage colony stimulating factor
gB	glycoprotein B
gC	glycoprotein C
gD5-23	synthetic peptide of specific epitope
H1A	Th1 - like producers
HSK	Herpetic Stromal Keratitis
HSV-1	Herpes Simplex Virus type 1
HSV-1 KOS	induces CD8+ T-cell infiltrates
HSV-1 RE	induces CD4+ T-cell infiltrates
LPS	lipopolysaccharide, component of gram neg. bacterial cell wall
MLN	mediastinal lymph nodes
mAB	monoclonal antibodies
mRNA	messenger RNA
NK	natural killer cell
P55	human tumor necrosis factor receptor

PECAM-1	vascular endothelial adhesion molecule
PMN	polymorphonuclear leukocytes
RT-PCR	reverse transcriptase - polymerase chain reaction
SCID	strain of mice with severe combined immunodeficiency
TCR-MHC	T-cell restricted major histocompatability complex
Th1 /Th2	CD4+ subsets
T-minus	mice depleted of T-cells
U1A	Th2 - like producers

PART I :

LITERATURE REVIEW

HERPETIC STROMAL KERATITIS

Ocular infection with herpes simplex virus (HSV) leads to an inflammatory lesion in the cornea called herpetic stromal keratitis (HSK). In the murine model, HSK is characterized by epithelial infection, edema, and epithelial and stromal destruction, leading to opacification and blindness. Some strains of HSV in susceptible strains of inbred mice causes HSK, stressing the genetic interactions between pathogen and host in this process [1]. One of the most fundamental observations in this field was reported by Metcalf et al.[2] These authors reported that ocular HSV infection did not cause HSK in nude (T-cell depleted) mice. However, adoptive transfer of HSV-immune whole splenocytes (minus adherent cells) caused ocular damage in nude mice upon infection [3]. Later, Russel et al. reported that HSK could be transferred to nude mice using HSV-immune T-cells, thus confirming Metcalf's findings [4]. The authors reported that HSK occurred in BALB/c mice after infection of the scarified cornea but similar reactions did not occur in athymic mice. However, if athymic mice were adoptively transferred with lymphoid cells, a severe necrotizing and ulcerative keratitis accompanied by corneal scaring resulted. These lesions progressed more quickly in recipients of lymphoid cells specifically immunized to HSV. In such mice, marked necrosis and ulceration were noted on the sixth day after transfer, compared with nine to twelve days for those given non-immune cells. Removal of T-cells from the immune lymphoid population by treatment with specific antiserum and complement abrogated the adoptive transfer of the stromal reaction. This data demonstrates that HSK represents a host immunopathologic response to HSV infection in which T-lymphocytes are essential participants.

The role of T-lymphocyte subsets in ocular immunopathology was evaluated by Newel et al. [5], who depleted CD4+ or CD8+ cells in vivo. CD4+depleted but not CD8+ depleted BALB/c mice were resistant to induction of HSK upon ocular HSV infection. Interestingly, in CD8+ depleted mice, HSK developed more rapidly, more severely and involved a greater percentage of the mouse population. These observations implicated the CD4+ T-lymphocyte subset as the principal mediator of HSK, and CD8+ T-cells as possible regulators that control the severity of HSK. The importance of CD4+ T-cells in HSK development was subsequently shown by Doymaz and Rouse [6], and Mercadel et al.[7]. In these experiments, HSK was transferred to thymectomized and T-cell depleted BALB/c or severe combined immunodeficiency (SCID) mice only by the CD4+ and not by the CD8+ subset. Doymaz and Rouse used an adoptive approach in which thymectomized and T-cell depleted mice (T-depleted) were reconstituted with different numbers of syngeneic immune T-cells after ocular infection with HSV-1. Although T-minus mice developed initial epithelial inflammation, HSK did not occur. Reconstitution experiments revealed that mice receiving unfractionated immune T-cells or CD8+ depleted populations induced significant HSK. In contrast, mice that received similar or increased numbers of cells depleted of CD4+ T-cells did not develop HSK. Histopathologic examination of the diseased corneas demonstrated that the stromal inflammation in athymic normal or T-minus reconstituted mice was characterized by extensive polymorphonuclear (PMN) leukocyte infiltration.

Mercadal et al.[7] used SCID mice for their studies. They analyzed the development of HSK in SCID mice reconstituted with different T-cell subsets. Corneal infections in SCID mice and cell - reconstituted SCID mice were investigated. Where as unreconstituted SCID mice failed to

develop HSK and died of encephalitis, mice reconstituted with T-cells developed severe lesions of HSK. T-cells of the CD4+ subset were found to be essential mediators of the HSK lesion, while T-cells of the CD8+ subset protected mice from the lethal infection. These data confirmed reports by Newel et al.[5] and provided further evidence that HSK represents an immunopathologic process mediated mainly by CD4+ T-cells.

WHICH SUBSET OF CD4+ T-CELLS ARE INVOLVED IN HSK

Although the predominant roles of CD4+ T-cells in HSK are well accepted by most investigators, there is no real consensus concerning the interactions of the different subsets of CD4+ T-cells (Th1/ Th2) in the development of disease. Niemialtowski and Rouse [8] reported that the cells isolated from infected corneas and restimulated in vitro with HSV produced mainly Th1-type cytokines. Niemialtowski and Rouse isolated cells from eyes in the active phase of HSK and studied their cytokine profiles after culture in vitro, or stimulation with HSV antigen (Ag), or nonspecific mitogens. After stimulation in vitro with HSV Ag or mitogens, the cytokines interleukin-2 (IL-2), interferon gamma (IFN-gamma) and tumor necrosis factor alpha (TNF-alpha) were produced [cytokines interleukin -4 (IL-4) or interleukin -10 (IL-10) were not however produced]. At the same time, Hendricks et al. [9] reported a dominant role for IFN-gamma and IL-2 (Th1 cytokines) in the process of HSK. These investigators reported that treatment of HSV-1- infected mice with monoclonal antibodies (mAB) to IFN-gamma or IL-2, when initiated one day prior to virus infection or six days after virus infection, caused significant reduction both in the incidence of stromal inflammation and the severity of inflammation, in those corneas that did develop disease. Treatment with mAB to IL-4 did not affect significantly the incidence or

severity of stromal inflammation. However, Jayaraman et al. [10] reported that transfer of a CD4+ Th2 clone induced HSK in susceptible mice. These authors generated a CD4+ Th2 clone from HSK -susceptible mice immunized with a synthetic peptide, gD5-23 which corresponds to amino acid number 5-23 of glycoprotein-D of HSV-1, constituting the T-cell epitope of gD of HSV-1. This T-cell clone produced and used IL-4 as an autocrine growth factor. Furthermore, gD5-23 specific T-cell proliferation of this clone was blocked by addition of anti- IL-4 but not anti-IL-2 antibodies. In addition, this T-cell clone failed to produce IFN-gamma or TNF-alpha. These authors reported that adoptive transfer of these gD-specific Th2 clones into HSK- susceptible mice accelerated strikingly the onset of HSV-mediated keratitis. To clarify this issue, Babu et al. [11] used a more sensitive approach, reverse transcriptase - polymerase chain reaction (RT-PCR). They isolated RNA from HSV infected corneas at the onset or during peak lesions of HSK. The conclusion of these experiments confirmed the predominant role of Th1 in HSK. These authors reported that during the onset and peak expression of HSK, type1 cytokines IFN-gamma and IL-2 levels were predominant in the corneas, and IL-4 levels were either very low or undetectable. Neither IL-10 nor IL-5 mRNA was detected during the course of the disease. Kanangat et al. [12] subsequently reported that IL-12 [12], which is involved in the differentiation of Th1 subset, is produced soon after HSV infection. The early induction and continued maintenance of IL-12 (P40) mRNA and protein in the cornea and draining lymph nodes upon infection with HSV was considered to be the triggering event in the immunopathology of HSK. This lead to the notion that after HSV infection, IL-12 is produced by infiltrating cells in the cornea which help the development of CD4+ Th1 cells. During the same period, the protective role of Th2 cells against ocular inflammation was confirmed by Babu et al. [11], and Tumpey et al. [13]. The former

reported that spontaneous recovery from HSK was associated with production of IL-10, and the latter injected IL-10 protein both systemically and stromally to protect ocular tissues from corneal damage caused by HSV.

POSSIBLE MECHANISMS OF CD4+ Th1 MEDIATED IMMUNOPATHOLOGY IN HSK

As HSK was shown to be the result of an immunopathologic destruction of corneal tissues, where the T-cells are the major participants, various authors attempted to identify and assess the cytolytic T-lymphocyte (CTL) activity in this process of ocular tissue destruction. Hendricks et al. [14] used mutant HSV to influence cell mediated immunity (CMI), and evaluate the modulation of CMI as it relates to the susceptibility to HSK development. These authors demonstrated previously that anterior eye chamber (AC) injection of HSV-1 before, or simultaneously with, topical corneal HSV-1 infection resulted in cellular immune tolerance to HSV-1, and a reduced frequency of corneal stromal lesions. Hendricks et al. [14] used a topical corneal infection with wild type HSV-1 followed by AC injection of the same eye with either wild-type HSV-1 or a mutant strain. A mutant strain lacking glycoprotein-C (gC) induced partial systemic tolerance, characterized by undetectable cytotoxic T-lymphocytes (CTL) activity, and a normal delayed- type hypersensitivity (DTH) response. The mutant strain lacking glycoprotein B (gB-) caused partial suppression of the CTL response and no reduction of the DTH response. Interestingly, the unique pattern of cellular immune reactivity exhibited by the mice receiving simultaneous topical corneal infection with wild-type HSV-1 and AC injection of gC- (no CTL, but normal DTH) was associated with significantly reduced susceptibility to HSV-1 corneal stromal lesions. In contrast, partial reduction of the CTL response by AC

injection of gB- HSV-1 did not render mice less susceptible to HSK. The authors concluded from these results that HSV-1-specific CTL may play an immunopathologic role in the development of HSK, although when using the HSV-1 KOS strain, the majority of the T-cells infiltrating the cornea are CD8+ cells. Therefor it was not established if CD4+ cells which are recruted to the cornea after HSV-RE infection, play a role in the CTL of HSK. Doymaz et al. [15] investigated the presence of cytotoxic CD4+ T-cells in the cervical and retropharyngeal lymph nodes of BALB/c mice experiencing HSK after ocular infection using HSV-1 RE strain. Their results showed the presence of a distinct CTL population which was CD4+ and which demonstrated lytic activity for the target cells infected with HSV-1, in a class II -restricted fashion. Furthermore, immunohistochemical analysis showed the presence of both CD4+ lymphocytes and I-A+ cells in the corneal tissues at the onset and the peak of HSK. The authors considered these findings supportive of the notion that direct lysis of infected class II - bearing corneal cells by CD4+ CTL might be one of the mechanisms leading to stromal immunopathology in HSK.

Niemialtowski and Rouse [16] investigated this issue more directly, to discover if cytoxicity was involved in the immunopathology of HSK by investigating the nature of inflammatory cells in the eye. They isolated various ocular cells from HSV infected corneas to establish their phenotypes and determine some of their functions. By means of immunocytochemistry and cytofluorography, the only T-lymphocyte subset detected at any stage of infection in BALB/c mice was the CD4+ type. However, CD8+ T-cells were readily detected in the draining lymph nodes (DLN). Assays for cells with HSV-1 - specific cytotoxic function activity were performed. Although in DLN both cell types were found, among ocular cells only CD4+ cytotoxic cells were evident. The

frequencies of CD4+ CTL-precursors in eyes were determined and found to be at least eight to ten fold less than those levels found in DLN. The number of CTL-precursors in an individual eye was estimated to be twenty or less. These results further supported the notion that CD4+ T-cells mediated the immunopathology of HSK, but based on their quantitative data, these investigators considered CTL activity to be an unlikely mechanism of tissue damage.

Although a predominant role of the CD4+ Th1 subset in the pathogenesis of HSK appears evident, it is not yet clear how these CD4+ Th1 cells are involved in ocular damage. Some possible mechanisms were proposed by Hendricks' group [17]. Mice were infected ocularly with HSV-1, one and ten days later they were treated with monoclonal anti- IFN-gamma. Treatment of mice 10 - 14 day post- infection with an IFN-gamma monoclonal antibody decreased the severity of disease compared to control groups. Within 48 hours after IFN-gamma antibody treatment, there was a marked reduction of neutrophil infiltration into the perivascular space. This reduction in neutrophil infiltration correlated temporally with a marked decrease in expression of the adhesion molecule PECAM-1 (platelet endothelial cell adhesion molecule 1 expressed mainly in vascular endothelium) [17]. The expression of this adhesion molecule was strongly up-regulated in the infected corneas with 2+ inflammation scores (10 -14 days post infection). Within 24 hours after anti-IFN-gamma treatment of mice with 2+ corneal inflammation, PECAM-1 expression on corneal vascular endothelium was noticeably reduced. By 48 hours after treatment, PECAM-1 expression was reduced to a level that is constitutively expressed in normal corneas. To confirm this finding that IFN-gamma regulates PECAM-1 expression on vascular endothelium in vivo, 100 units of recombinant mouse IFN-gamma were injected into normal mouse corneas, and the corneas were monitored for PECAM-1 expression by

immunohistochemistry and image analysis. PECAM-1 expression was strongly up-regulated 3 hours after injection of recombinant IFN-gamma. The authors concluded that IFN-gamma, a Th1 cytokine, contribute to HSV-1 induced corneal inflammation by facilitation of neutrophil infiltration as a consequence of PECAM-1 up-regulation on the vascular endothelium. Recently, the same group focused on the role of IL-2 [18], another of the Th1 cytokines involved in the pathogenesis of HSK. Their approach was to allow corneal inflammation to develop to various levels, and then to initiate neutralization of IL-2 with monoclonal antibodies in vivo. The authors reasoned that if neutralization of IL-2 resulted in a rapid alteration in the inflammation, the altered functions could be attributed directly or indirectly to IL-2. Intraperitoneal injection of IL-2 mAb at the 2+ stage of inflammation resulted in a marked diminution of corneal inflammation. The number of corneal IFN-gamma secreting cells was significantly reduced 48 hours after treatment, as was the level of IFN-gamma mRNA. In addition, the PMN chemotactic gradient in the infected corneas was abolished, and the PMN that were already present in the central cornea exhibited morphologic signs of apoptotic cell death [18]. This distribution of viable and apoptotic PMN suggested that IL-2 is required for the maintenance of a PMN chemotactic gradient in the infected corneas, and for the maintenance of PMN viability at the focus of inflammation in the central cornea. Based on these experiments, Hendricks et al [18] proposed a mechanism by which IL-2 and IFN-gamma, the Th1 cytokines, were involved in the resolution of HSV infection in mouse corneas. CD4+ T - lymphocytes are activated by HSV antigens presented on the Langerhans cells. The activated CD4+ T-cells produce IL-2 and IFN-gamma. IL-2 acts on the CD4+ T-cells in an autocrine or paracrine fashion to maintain IFN-gamma production and high levels of IFN-gamma up-regulate PECAM-1 expression on the vascular endothelium. PMN

interact with PECAM-1 and extravasate into the HSV-infected corneas. IL-2 and IFN-gamma activate PMN, protecting them from apoptotic death and increasing their exocytosis of collagenases and other proteolytic enzymes, contributing to the destruction of corneal architecture.

THE ROLE OF NEUTROPHILS IN HSK

In the last decade there has been a major focus on the T-cell immune response in HSK. In the process, this concept addresses the importance of innate immune response. For more than twenty years it was noted that the innate immune response was a part of the pathophysiology of HSV infection [19]. Although it was noted that neutrophils are the predominant cell type infiltrating the cornea during HSK [20], their role was not evaluated until recently. This oversight on the part of researchers regarding the delineation of the role of PMNs was mostly due to the concept that these cells had limited capacity to produce a variety of proteins. Also, that their major defensive role was against bacterial infection where PMN have been studied in greater detail.

Involvement of PMN in HSV growth in vivo and process of HSK was evaluated by Tumpey et al. [21]. The experimental approach was to treat mice with monoclonal antibodies specific for Gr-1 surface Ag expressed on murine granulocytes, to deplete mice of PMN and then to test the effect of this on virus replication in the eye and in the peripheral and central nervous system. The virus titers in the eye were significantly higher than those in the control groups at 3 days post-infection. By day 9, virus was no longer detectable in the controls, whereas titers of nine thousand to one million pfu were still present in the neutrophil-depleted hosts. Furthermore, virus spread more readily to the skin and brains of the treated mice, rendering them significantly more susceptible to HSV -1-

induced blepharitis and encephalitis. Only 25% of the treated animals survived, where in contrast, all of the control mice lived. Similar results were obtained in SCID mice. This indicates that in both immunocompenant and immunodeficient mice, neutrophils play a significant role in controlling the replication and spread of HSV -1 after corneal infection. Thomas et al. [22] characterized in more detail the role of the PMN in HSK. They reported that the early neutrophil infiltration lasted for 48 - 72 hours, and then 3 days after infection, few neutrophils were detected as this inflammatory response wanes. However, a second, more massive neutrophil infiltration occurred, beginning at 8 to 9 days post infection, this coincided to when clinical HSK became most evident. The role of the PMN in the development of HSK was investigated using SCID mice. In SCID mice, where HSK does not occur, unless they are adoptively transferred with CD4+ T-lymphocytes, the effect of neutrophil depletion was more pronounced in SCID mice reconstituted with the CD4+ T-cells. There was a lower incidence and decreased severity of HSK in CD4+ T-cell- reconstituted SCID mice that were depleted of neutrophils as compared with controls. Neutrophil- depleted mice displayed moderate to severe periocular skin lesions, progressive cachexia, and developed signs of encephalitis. Virus was recovered in higher titers and for longer periods from eyes of neutrophil-depleted animals. Brain viral titers were also significantly higher on day 12 post- infection as compared with the SCID mice which were not depleted of PMNs. The authors suggested that herpes simplex virus infection of the cornea rapidly invoked recruitment of PMNs that might aid in viral clearance, and that they directly or indirectly served as agonists in perpetuating a CD4+ T-cell- mediated inflammatory reaction. Bouley et al. [23] investigated the effect of natural killer (NK) cells in the reconstituted SCID mouse model of HSK, they confirmed the importance of PMNs in this system. The

effect of NK cell depletion appeared to regulate the number of PMN that migrated into inflamed corneas. In the absence of NK cells, there were significantly fewer corneal PMN and less severe clinical and histopathological HSK lesions.

Th1 AND Th2 CONCEPT

In 1986, Mosmann and colleagues reported that murine CD4+ T-helper cells could be polarized into two different subsets: Th1, CD4+ T-cells producing IL-2 and IFN-gamma, and Th2, CD4+ T-cells producing IL-4, IL-5 and IL-10. Since this discovery, a large body of immunology research has dealt with this notion, and even the basic researchers in the other biological disciplines became interested in this subject. The interest of immunologists is based mainly on the observation that, in a large number of bacterial, viral and fungal infections, etc., the induction of disease and it's progression is associated with the predominant presence and involvement of one specific type of CD4+ T-helper cell (Th1 or Th2 depending on the disease process). It was noted that, cure and recovery from these inflammatory diseases were often associated with the inverse subset predominating. These immunologists were intrigued by these discoveries and driven to understand the molecular differences between Th1 and Th2, apart from the different cytokine secretion patterns and the signals which drive them into the different pathways. Knowing the importance of these two subsets in the progression of disease, the question arises: if these two populations could be reversed then what would be the consequences when one population shifts toward the other? This question leads to the complex area of the regulation of homogeneity or heterogeneity in these populations. Murphy and colleagues [24] reported that once the Th1 or Th2 clones are established in culture (long term clones) the cytokine pattern of expression of these Th1 or the Th2 clones

could not be reversed [24]. However, there is in vivo data suggesting that Th1 or Th2 responses following pathogen infection or antigen (Ag) administration might be reversed under certain circumstances [25]. These data favor the notion that even the global response of the animals correspond to high expression of one subset of T-helper cells and low expression of the other subset, in vivo there is either a mixed population of these two subsets (high numbers of one subset and low numbers of the other subset) or there is one subset of T-helper cell with different stages of activation and differentiation. It was suggested that IL-12 receptor (IL-12 R) Beta-2 subunit, the binding and signal transducing component of IL-12 R, is expressed in Th1 and not in Th2 clones [26]. This observation was reported as the basis of Th1 / Th2 differentiation. The necessity of IL-12 to promote Th1 development was shown previously using IL-12 P40 -/- and STAT 4-/- (signal transducer and activator of transcription 4 protein necessary for IL - 12 action) mice [28]. In these two knock-out mice there was nearly a complete inability to generate a Th1 response. Zheng et al. [29] performed cDNA subtraction analysis and reported identification of transcription factor GATA3, which is necessary and sufficient for cytokine gene expression profile in CD4+ Th2 cells [29]. GATA3 is a member of a multi-gene zinc finger transcription factor which recognizes a common consensus motif (WGATAR). GATA3 is most abundantly expressed in T-lymphocytes [17]. Based on this new finding and the previous knowledge, these authors postulated a model for Th1 / Th2 generation. In this model, following antigen stimulation, the T-cell type that produces high levels of IFN-gamma and IL-4 (Th0) is generated with it's intrinsic properties to be polarized toward the Th1 or Th2 subset. The Th0 cells possess high levels of GATA3 (necessary for Th2 generation), and high levels of molecule X (necessary for Th1 generation) [29], along with the presence of different cytokine receptors. In an environment where the IL-4 concentration is

much higher than that of the IL-12, (such as in draining lymph nodes) then TCR - MHC interactions and IL-4 and its receptor generate intracellular signals leading to complete inhibition of factor X and maintenance of high level expression of GATA3. Once these cells lose their membrane-bound IL-12 receptor they become resistant to IL-12 action. At the same time, high levels of transcription factor GATA3 continue to induce the expression of Th2 type cytokines. However, if the Th0 cells find themselves in an IL-12 environment, upon IL-12 action, there is high expression of factor X and a decreased expression of GATA3 in these cells. Factor X induces expression of Th1 cytokines, and because of lack of GATA3, there is no Th2 cytokine production leading the cell to a Th1 subset. This concentration balance between factor X and GATA3 will fix the cells into either the Th1 or Th2 phenotype. In the Th1 cells, one can speculate a positive autoregulatory loop for factor X production and at the same time this factor can suppress to a basal level expression of GATA3. In the Th2 cells the situation is reversed, where it is possible that a high level of GATA3 directly or indirectly suppresses the factor X expression which is necessary to switch to Th1. This concentration balance between the two proteins could give a molecular explanation to the possible existence of intermediate CD4+ T-helper cells between Th1 and Th2 extremes that express mixed sets of cytokines. But, what are the factors which induce the expression or suppression of these different intracellular proteins which in the end, result in development of Th1 or Th2? Multiple factors have been proposed to be involved in the development of different subsets of T-helper cells. How important these factors are, especially in combination with each other, is not well understood or defined. A list of factors would include Ag, dose of Ag, cytokine environment where the T-cells are primed, route of infection, type of immunogen (DNA vs. protein), co-stimulatory molecules, genetic background, hormones, type of antigen

presenting cells (APC), sequence of Ag (altered peptides) etc.[30]. The factor that is the most studied is the cytokine environment, usually looking at the draining lymph nodes or the effector tissues, and this has been the basis for the generation of a defined milieu for the modulation of inflammatory disease by direct administration of different cytokines.

Th1 AND Th2 IN DISEASE

Cytokines play a pivotal but paradoxical role both in immunity and inflammation. These local inflammatory peptides form a major arm of the animal's defenses against infectious organisms, but they are also implicated as potent mediators of the pathophysiology of infectious disease [31]. Herpetic stromal keratitis (HSK) is reported to be associated with a Th1-type response but this viral ocular infection is one of the many infections or autoimmune diseases where Th1 or Th2 are reported to be involved in the establishment of disease and it's maintenance.

Experimental allergic encephalomyelitis (EAE) is one of the best studied models for the involvement of T-helper cells in the development of disease [32]. EAE is an autoimmune disease of the central nervous system that serves as a laboratory model for the human demyelination disease, multiple sclerosis. In mice, effector CD4+ Th1 cells respond to myelin antigens and secrete proinflammatory cytokines IL-2, IFN -gamma, and TNF-alpha. The importance of the Th1 cytokines in the establishment of EAE was understood by using neutralizing Ab to different Th1 cytokines to inhibit inflammation [33]. On the other hand, adoptive transfer of antigen-specific Th2 cells or direct administration of Th2 cytokines resulted in protection from disease [34]. This protection was abrogated if antibodies to IL-4 were administered at the same time. Horwitz et al. [35] showed the crucial role of IFN-gamma, a Th1 cytokine, in the

demyelination process, which is the pathogenic feature of EAE, by using transgenic mice expressing IFN-gamma in the central nervous system. The authors generated a transgenic mouse that expressed murine IFN-gamma in the central nervous system by using an oligodendrocyte-specific promoter. Associated directly with the expression of the transgene (after eight weeks of age) primary demyelination occurred and was accompanied by clinical abnormalities consistent with central nervous system disorder. Even though CD4+ Th1 cells are reported to be the major participants in the pathogenesis of EAE, it is important to note that when the Th1 cytokines (TNF - alpha and IFN- gamma) are knocked-out, EAE can still be induced in these animals [35]. However, this does not mean that these factors are not important or not involved in the induction of EAE. We can speculate that once a cytokine is knocked-out, it's function could be overtaken by other cytokines (redundancy of function) and the animal adjusts itself to the absence of this factor.

Leishmania major (L.major) infection is another model where the importance of Th1 / Th2 was studied [36]. L. Major infection of BALB/c mice results in progressive parasite replication and spread to macrophages in the visceral organs with a uniformly fatal outcome. Susceptible strains of mice (example BALB/c) develop poor cell-mediated immunity to the parasite and produce cytokines associated with a T-helper type 2 response, including IL-4, IL-5, and IL-10 following infection. Similarly, enhanced levels of IL-4 and IL-10 were reported in human patients with non-healing forms of leishmaniasis [37]. In contrast, resistant mouse strains such as C3H, develop Th1 responses, characterized by cell-mediated immunity and INF-gamma production. Susceptible BALB/c mice could be rendered resistant with various immunologic treatments influencing Th1 / Th2 balance including treatment with anti-IL-4 [38], anti-TGF –B [39], or recombinant IL-12 [40]. Such treatments were only effective if

manipulation started at or near the time of L. major infection. However, Nabros et al. [41] reported that even in established L. major infection, mice could be cured by combined therapy with IL-12 and pentostam, a pentavalent antimony compound that reduces the parasite burden. Adoptive transfer experiments, using either Th1 or Th2 clones producing Th1 or Th2 cytokines, were able to show the importance of Th1 / Th2 in the course of the disease. Holaday et al. [42] established two different cell lines from the draining lymph nodes of BALB/c mice infected with L. major, a Th1 like line, H1A, and a Th2 - like cell line. U1A. Mice with severe combined immmunodeficiency (SCID) were reconstituted with H1A or U1A before infection with L. major. Non- reconstituted SCID mice were unable to control parasite growth. Mice reconstituted with H1A (Th1 - like cell line) cleared infection, where mice reconstituted with U1A (Th2 - like cell line) suffered an exacerbation of disease.

IMMUNOINDUCTION AND IMMUNOMODULATION USING PLASMID ENCODING ANTIGEN OR CYTOKINES

Accepting the above data that the pattern of cytokine production during different inflammatory responses could be involved in the development of disease, and that the Th1/Th2 response could be involved in the treatment of disease, it becomes tempting to try to deliver these cytokines directly in an effort to modulate the immune response, and therefor the outcome of disease. Even though this approach has been used with success in many animal models of inflammatory disease [40], it has its own limitations : First, proteins, especially cytokines, have short half-lives. Even though several investigators overcome this handicap by linking their protein of choice to human or mouse IgG to increase the half-life, this is still an obstacle; second, large scale production and safe handling of proteins are problematic and, third, the local delivery of cytokines is a

major handicap. For example, it is difficult to administer an intracranial injection of IL-4 for the treatment of multiple sclerosis.

Use of viral vector as a delivery system for cytokines is an alternative methodology. In this case, safety considerations, the interaction of the host immune system with the virus and viral tropism are major handicaps. Accepting Schrodinger's assumption that "genes are the most important compounds of a living cell", it is possible to use the corresponding gene cDNA, of a cytokine and deliver it to the site of inflammation via a nucleic acid vehicle (plasmid DNA) to fight against viral or bacterial invasion.

GENE DELIVERY TO GENERATE IMMUNITY

Considerable amounts of data have been generated since 1992 that show that injection of naked plasmid DNA encoding an antigen can induce an immune response. Many of the advances in this area relied on the previous 30 years of development of molecular biological techniques. It was much easier for an investigator to prepare a plasmid encoding a viral glycoprotein than it was to purify that glycoprotein in large amounts. The technique of plasmid preparation was convenient and it allowed for large scale DNA preparation for the utilization of different variable combinations such as Ag, cytokine, costimulatory factors etc. to study and understand the generation of immune response and the cell biology of this process. Accepting the concept that plasmid DNA delivery helps to understand the immune response, there's no need for an affirmative response to the question "Can genetic immunization be used in humans?" to justify the continued support and further experimentation in this field .

In 1992, Tang et al. [43] reported that plasmid coated onto gold beads and delivered into muscle drove the expression of foreign protein

and stimulated an antibody response. In 1993, several groups reported that naked DNA plasmids (those without a delivery vehicle) encoding a viral or bacterial protein, generated protective immunity against such a pathogen [44]. A list of pathogens in which genetic immunization has been successful includes : the human immunodeficiency viruses 1 and 2 (HIV 1 , 2), herpes simplex virus 1 (HSV -1), influenza virus, rabies, tuberculosis and malaria. In these experiments, a plasmid DNA encoding a protein corresponding to the pathogen was administered mainly intramuscularly (IM). The expression of the plasmid protein was in general, driven by a viral-specific promotor that is condensed, strong and ubiquitous, to allow sustained expression of foreign proteins. Even though the viral promoters are of the first choice, plasmids containing cellular promotors were also effective way to drive the expression of foreign proteins with subsequent generation of an immune response.

HOW PLASMID DNA INDUCES AN IMMUNE RESPONSE

One of the most interesting questions in genetic immunization is to determine or definition how the immune response is generated after DNA administration. Even though different routes for gene delivery have been used, the most studied is IM administration of plasmid DNA. Upon IM injection, antibodies (Ab) and T cell immune responses (as measured by lymphocyte proliferation , CTL, DTH and cytokine production) are generated. But, in which cells are the proteinsexpressed after IM immunization? Most studies use plasmids encoding B-galactosidase (B-gal) to identify the cell expressing the foreign protein [45]. In such experiments, after staining the muscles for B-gal activity, myoblasts were identified as the major site of foreign protein expression. Accepting that the major site of protein production in response to in vivo transfection is the muscle cell, we are confronted by an enigma, because myoblasts are

virtually devoid of MHC class II expression. Also, they express very low levels of MHC class I antigens, and lack the costimulatory signals (B7-1, B7-2) for T-cell priming [46].

Some light was shed on this issue using transfer of bone marrow cells expressing one MHC haplotype to irradiated F1 chimeric recipient mice [47]. These mice were injected intramuscularly with naked plasmid DNA encoding a single antigen containing epitopes for both MHC molecules. These experiments revealed that CTL responses were restricted to the MHC haplotype expressed by the bone marrow alone, and not to the haplotypes expressed by the recipient myocytes. Then what is the role of myoblasts in immune induction? There are different hypotheses to explain this issue. One of the most appealing hypotheses is to the consideration of the somatic tissues (myoblasts) as a reservoir for expression of the foreign protein. Once the protein is produced, it is secreted out of the cells and taken by the antigen presenting cells (APC). However, a direct injection of plasmid DNA into the APC in these experiments can not be ruled out. The theory favored by H.C. Ertl [48]. Accordingly :

" the most likely pathway for initiation of an immune response by genetic immunization is the direct transfection of professional antigen presenting cells. The number of Ag - presenting dendritic cells needed to initiate an immune response is minute, and lack of histological evidence for direct transfection of dendritic cells might reflect either that their number is below the threshold of easy detectability or, alternately, that they rapidly leave the site of inoculation and migrate to the draining lymph nodes."

Ertl strengthened her argument with S.A. Johnston's [48] findings that surgical removal of the site of DNA inoculation failed to abrogate the immune response. She alludes to her own findings that granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that activates

dendritic cells, improved the T-helper cell response to genetic immunization as another support of direct involvement of APC in this process [49]. Experiments conducted by Rogy et al. [50] add another level of complexity concerning expression of foreign proteins. These authors reported that 48 hours after intraperitoneal (IP) injection of DNA encoding human tumor necrosis factor receptor (P55) or IL-10, both human P55 and IL-10 mRNA expression was observed in the spleen, liver, kidney and the lung. However, recently, D. Weiner's group reported the data supporting a direct role for the myoblasts. This group reported that coadministration of B7-2 along with HIV-1 gag/pol DNA resulted in a dramatic increase in cytotoxic T-lymphocyte proliferation [51] which strengthens the role of the myoblast. Immunohistochemistry analysis of injected muscle revealed that costimulatory molecules B7-1 or B7-2 were expressed in myoblasts after intramuscular injection. Do these results mean that muscle cells were being engineered to function as APC by enabling the expression of costimulatory molecules? Although the induction of immune responses using plasmid DNA is now widely accepted, it is not at all clear how this phenomenon occurs. It seems as if better manipulation of the immune response using plasmid DNA will be an area of future research.

DISEASE TREATMENT USING PLASMID DNA ENCODING CYTOKINES

In 1993, E. Raz et al. [52] reported that humoral and cellular immune responses to keyhole limpet hemocyanin (KLH) and transferrin proteins could be modulated by plasmid DNA encoding IL-2, IL-4, and TGF-B1. These data emphasized the potential of using plasmid DNA encoding cytokines in vivo. The same group reported that antibody production after immunization of mice with plasmid DNA encoding an isolated human Ig variable region was enhanced by a co-transducing IL-2

encoding plasmid [53]. However, Abai et al. [54] reported that a plasmid encoding IL-2 did not increase the humoral immune response to the antigen encoded by another plasmid. In 1995 Xiang and Ertl [49] published that coinoculation with vector expressing mouse GM-CSF along with a plasmid encoding rabies virus glycoprotein, enhanced the B and T helper activity to rabies virus protein.

"The enhancing effect of the GM-CSF expressing vector on the immune response to the G - protein-encoding vector depended on coinjection of the two plasmids, inoculation of the two plasmids separately several hours apart, had no effect on the magnitude of the antibody response to rabies virus, suggesting that either cotransfection of individual cells with both vectors or close proximity of APC's to GM-CSF secreting cells was crucial, presumably reflecting the localized activity of the cytokine."

However, one of the first reports dealing with the successful use of plasmid DNA encoding cytokines and treatment of disease was published in 1995 by M. Rogy and colleagues [50]. These authors reported that intraperitoneal injection of plasmid DNA encoding tumor necrosis factor receptor (P55) or IL-10 in the mouse, reduced mortality to lethal endotoxemia when challenged with LPS and also attenuated the local inflammatory response to LPS. In these experiments, mice were treated intraperitoneally with cationic liposomes containing 200 ug of either a P55 expression plasmid containing the extracellular domain and transmembrane coding region of the human P55 TNF receptor, or an IL-10 expression plasmid containing the cDNA for human IL-10. Pretreatment of mice with P55 or IL-10 cDNA -liposome complexes improved survival to LPS. In addition, intratracheal administration of IL-10 DNA-liposome complexes 48 hours before an intratracheal LPS challenge reduced pulmonary TNF-alpha levels by 62% and decreased neutrophil infiltration in the lung by 55%.

Treatment of allergic asthma by plasmids encoding cytokines was another example of treatment of disease by cytokine gene delivery [55]. Three days after Ag challenge, conalbumin-sensitized mice received intratracheal instillation of 20 ug of plasmid encoding IFN-gamma compounded with liposomes. This led to a significant decrease in the number and percentage of eosinophils in bronchoalveolar lavage fluid. This was associated with a reduction of conalbumin-induced airway hyperreactivity to acetylcholine. Li et al.[55] reported that following intratrachial mucosal delivery of plasmid DNA, the expression of encoded protein was localized in the pulmonary epithelium, but absent in the spleen or liver. These authors also reported that mucosal IFN-gamma gene transfer had a pronounced inhibitory effect on the level of Th2 cytokines, leading to a marked decrease in pulmonary eosinophil infiltration.

The two above examples dealt with the use of cytokines in inflammatory diseases, however, Rakhmilevich et al. [56] reported the use of an IL-12 expression plasmid for cancer treatment. In this system, the two subunits of IL-12 were expressed from the same plasmid which contained two cytomegalovirus promotors. Transfer of IL-12 cDNA in epidermal cells overlying an implanted intradermal tumor resulted in detectable levels of the IL-12 protein at the epidermal treatment site, and the complete regression of established tumors. In addition, the authors further showed that injection of IL-12 cDNA into the skin overlying an advanced intradermal tumor, followed by tumor excision and three additional IL-12 gene injections into the same location, significantly inhibited systemic metastases, resulting in extended survival of test mice.

PERSPECTIVE AND FUTURE STUDIES

Generation and manipulation of the immune response was found to be an effective way of combating different pathogens. Multiple approaches are used to generate a strong and sustained protective immunity. Even though prophylactic treatment has been successfully applied to multiple disease processes, there are still many improvements necessary for effective application of these methods in the fight against multifaceted pathogenic agents. The methodology that deals with the established infection, and therapeutic treatment of disease will probably be the focus of future investigations. But, how can one stop or reverse the course of disease once the inflammatory parameters are set? There is room for the imagination in this process, and the most successful approach will be a combination of various treatment modalities. We hypothesize that somatic gene delivery may be a novel approach for overcoming established infections if the delivered genes are expressed for a sustained time, and if the encoded proteins have some beneficial suppressive effects on the immune response. Our preliminary data showed that somatic gene therapy was a successful approach for the modulation of the immune response and could be employed successfully to fight ocular infection with Herpes Simplex Virus-1. This is significant because we have found that it is possible to modulate the immune response in vivo using plasmids encoding cytokines. The application may be even more important for those diseases where there are no early diagnostic tests available for the early identification / diagnosis of disease. This mode of therapy may be used for pathogens where there is no effective vaccine available. However, if administration of eukaryotic expression vectors have such a profound effect on immune system as to block ongoing inflammatory disease, it could be reasoned that its expression is not restricted to the site of administration of the plasmid. In fact, systemic immune suppression is

easy to visualize if you consider that after the administration of genetic material in a particular location, it can be expressed in different, distant sites. On the other hand, another potential problem is that during suppression of an ongoing inflammatory response, the subject could easily become a target for other opportunistic infections. It will be possible to overcome these handicaps if the expression of foreign DNA can be restricted to a defined tissue, and if its expression could be reversed by exogenous modulators. In conclusion, we can assume that having a tool capable of altering the immune response will help us to better understand the complexity of the immune system.

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PART II :

OCULAR NEUTROPHILS PRODUCE KEY MOLECULES INVOLVED IN THE PATHOGENESIS OF KERATITIS CAUSED BY HERPES SIMPLEX VIRUS INFECTION

PURPOSE

Polymorphonuclear cells (PMN) are the prominent cells infiltrating the cornea upon ocular HSV-1 infection. The author investigated the role of ocular PMN in production of pro-inflammatory molecules, TNF-alpha, (inducible nitric oxide (iNOS), and IL-12 at 24 hours following HSV-1 infection. The proinflammatory molecules TNF alpha, iNOS, and IL-12 were induced as a result of natural infection, one day following HSV-1 innoculation. These molecules were mainly localized in the corneal stroma of infected eyes, and absent in the corneal stroma of trauma controls. PMN were identified as the major source of these molecules. These results showed that the infiltrating cells, mainly the PMN, in the stromal cornea were the source of early production of proinflammatory molecules TNF-alpha, iNOs, and IL-12. However, the virus was localized in epithelial cornea, showing a physical separation between infected cells and cells producing inflammatory molecules.

INTRODUCTION

Almost immediately following infection of the mouse cornea with herpes simplex virus (HSV), there is an invasion of cells, almost exclusively neutrophils, in the underlying stroma [(1)]. These cells disappear in 3-4 days corresponding closely to the time when replicating virus is no longer detectable [(2)]. Indeed, viral clearance may be mediated by PMN, although if and how such an event occurs has not been proven [(3)]. Characteristically, after some days chronic inflammatory events occur in the eye and these appear to be orchestrated by CD4+ T lymphocytes that produce type 1 cytokines [(4)]. This HSK lesion also occurs in humans and is a common cause of blindness [(5)].

Since PMN invasion is such a prominent phenomenon, it was supposed that these cell may contribute to the direction of subsequent events. Conceivably PMN could be involved in the preferential induction of the Th1 CD4+ T cell response, clearance of virus and perhaps in the up-regulation of stromal host antigens that are suspected to act as additional targets for the HSK immunoinflammatory lesions [6]. In this report, we have employed in situ hybridization to establish if ocular PMN produce key inflammatory cytokines that would permit them to function in some or all of the aforementioned processes. These results show that ocular PMN produce TNF-alpha and iNos as is known to occur with PMN at other sites in the body, but they also produced IL-12. This observation, which was recently shown for PMN in another mouse model [7], could mean that PMN provides the major trigger for the Th1 dominated T cell response characteristic of HSK.

MATERIALS AND METHODS

Mice : Four to five week-old female BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and acclimated for 1 week before use in a specific pathogen-free animal facility. Experimental procedures used in this study confirmed to the resolution adopted by Association for Research in Vision and Ophthalmology, on the use and care of laboratory animals.

Virus : HSV-1 strain RE and HSV-1 expressing beta-galactosidase (a gift of Dr. E. S. Mocarski, Stanford, CA) were grown on monolayers of vero cells monolayers (ATCC CCL81) and were stored in aliquots at -80'C until used.

Corneal Infection : Mice were deeply anesthetized with methoxyflurane (Metofane, Pittman-Moore, Mondelein, IL), and corneas were scarified using a 27-g half-inch needle in a cris-cross pattern. 1 x 10^6 $TCID_{50}$ of virus in 4 ul volume was placed on the cornea and massaged with the eye lids.

Non-radioactive In-Situ Hybridization : The whole eye from trauma control and HSV infected mice was fixed in 4% paraformaldehyde in DEPC treated PBS for 4 hours at 4'C and kept in 70% ethanol until paraffin embedding. The 6 micron sections were washed in xylene and ethanol to remove paraffin. The slides were treated in 0.2 m HCl for 8 minutes, rinsed in 0.1 M triethanolamin-HCl, pH 8.0 for 5 minutes, treated with 0.1 M triethanolamin-HCl, pH 8.0, 0.25% acetic anhydride for 15 minutes and then washed in triethanolamine and rinsed with 2X SSC. The probes used were anti-sense or sense, single stranded RNA molecules produced by transcription from a T5, SP6, or T7 primer of a cDNA insert. 5 ng of digoxigenin-labeled probes were diluted in 30 ul of hybridization buffer containing 500 ul of formamide, 200 ul of 20X SSC, 20 ul of yeast tRNA (10 mg/ml) and 200 ul of 50% dextran sulfate for 1 ml volume.

The coverslides were incubated at 42'C overnight then washed in 2X SSC at room temperature (2 times), rinsed with STE buffer containing 500 mM NaCl, 1 mM EDTA and 200 mM Tris-HCl, pH 7.5. Sections were treated with 500 ul of Rnase A (100 ug/ml in STE) for 45 minutes at 37'C. The slides were washed serially in STE, 2X SSC, 1X SSC, 0.5X SSC and 0.1X SSC for 10 minutes each step at room temperature. Immunological detection were done using anti-digoxigenin-AP antibody (Boehringer Mannheim, Mannheim) after blocking the slides with 10% horse serum for 30 minutes. The slides were then washed in buffer I (100

mMTris-HCl pH 7.5 and 150 mM NaCl) for 5 minutes in buffer II (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 500 mM $MgCl_2$) for 10 minutes. Slides were covered with 500 ul of AP color reagent solution for 3 hours. AP color reagent solution contained 100 ul of AP color reagent A (Bio-Rad, CA), 100 ul of AP color reagent B (Bio-Rad, CA) and 2 drops of levamisole solution (Vector, CA) in 10 ml of Buffer II. Slides were serially dehydrated and mounted in paramount.

Immunochemistry and B-gal Staining : Eyes from traumatized or HSV infected mice were removed and fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 6 um, and stained with hematoxylin and eosin. For B-galactosidase staining, whole eyes from HSV-1 expressing B-galactosidase or control mice were removed and fixed in 4% paraformaldehyde in PBS, pH 7.2, for 1 h at 4'C. The tissues were washed several times in PBS and placed overnight in substrate solution containing 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM $MgCl_2$, 2 mg 5 bromo-4-chloro-3-indolyl-B-d galactoside (X-gal) per ml, and 10 ul of 10% nonidet P-40 and 100 ul of 1% sodium deoxydolate per 20 ml. The tissues were washed in PBS, fixed in 3.7% formaldehyde, embedded in paraffin, sectioned at 6 mm, and stained with nuclear fast red.

RESULTS

Pro-inflammatory cytokine producing cells at day one PI are mostly found in the stroma. To localize the presence of these proinflammatory mRNA in the cornea the paraffin embedded ocular sections were prepared from trauma or day one PI mice and hybridized with non-radioactive digoxigenin labeled sense or with anti-sense probes

corresponding to iNOS, IL-12 (P40) and TNF-alpha. The isolated positive signals were located in the stroma of HSV infected eyes using anti-sense riboprobe (Fig. 1) and no signal was detected in uninfected trauma controls (data not shown). None of the sense probe slides showed any signal using trauma or HSV infected ocular sections (Fig. 1). Histopathologic examination of the stroma at high power showed positive signal in cells with neutrophil characteristic using IL-12 (P40), TNF alpha and iNOS antisense riboprobes. In addition, there were positive cells other than neutrophils evident in the stroma but their identity was not was not characterized.

Localization of Virus and Neutrophils at Day One Post-infection : HSV-1 expressing B-gal was used to determine the localization of virus at day one post-infection. As shown in Fig. 2, the virus was exclusively localized to the epithelial cornea.

However, H&E staining of a day one infection section show the infiltrating cells, mainly the neutrophils in the stromal cornea. These data lead to the conclusion that there is a physical separation between the infected cells and pro-inflammatory producing cells.

DISCUSSION

Our report demonstrates that ocular neutrophils which infiltrate the eye as a sequel to HSV infection represent a source of three key molecules, IL-12, iNOs, and TNF alpha which likely influence subsequent events which culminate in the expression of herpetic stromal keratitis. This disease in humans is a common cause of blindness worldwide. Of particular interest was the observation that ocular PMN represent a source

of IL-12. Thus far, that PMN generate IL-12 appears not to have been documented. However, we [8] have recently shown that HSV infection leads to a rapid upregulation of IL-12 and this observation was repeated in the present communication. Of interest in the eye itself HSV replication and antigen expression appear to occur principally in the epithelium yet such cells showed little or no evidence for IL-12 production at least under conditions used for in situ hybridization in the present report. Instead, the signal was focused in the stroma and was assumed to be produced by PMN. Whether such cells produce IL-12 spontaneously with the cytokine representing a regular product of tissue PMN, or if such cells only produce the signal in response to stimulation by some HSV product is currently under investigation. Whatever the mechanism, our data are consistent with the notion that PMN may represent a primary source of IL-12 which helps influence the microenvironment to favor the induction of a CD4+ Th1 response.

It was also of interest to note the ocular PMN also represent sources of both iNOs and TNF alpha. Both molecules were shown previously in other systems to be products of tissue PMN. Of interest both TNF alpha and nitric oxide may be antiviral effects [9,10] and this could provide the means by which PMN exert antiviral activity. That such cells appear as antiviral has come from previous experiments by our laboratory as well as others showing that when the PMN response is suppressed, viral replication and persistence is more intense [2,3]. On the other hand, NO, product of iNOs is reported to be involved in enhancement of vascular permeability [11] and TNF alpha in neovascularization [12] and cell attraction [13], the key events in induction of HSK.

Finally, evidence exists which indicates that PMN infiltrates may release products which upregulate corneal autoantigens which then act as

agonists for the immunoinflammatory response. One such PMN product which may effect such an effect is nitric oxide. Clearly ocular PMN which express iNOs represent a potential major source of NO. We are currently using a variety of approaches including observations in iNos knock-out mice to determine the role of NO production in the pathogenesis of HSK.

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Figure 1: **In situ hybridization on HSV infected eyes using the non-radioactive digoxigenin riboprobes.**

10^6 PFU of HSV-1 (RE) were applied to the scarified eyes of BALB/c. 24 hours after, the whole eye was removed fixed in 4% paraformaldehyde, embedded in paraffin and sectioned (6 microns). Sense and anti-sense probes for TNF alpha, (A, B, C), iNOs (D, E, F) and IL-12 P40 (G, H, I) were prepared and hybridized over on different sections at 42'C. A, D, G sense probes (X250), B, E, H and G, F, I anti-sense probes (X250 or 625X respectively). The arrows show the positive cells with polymorphic morphology.

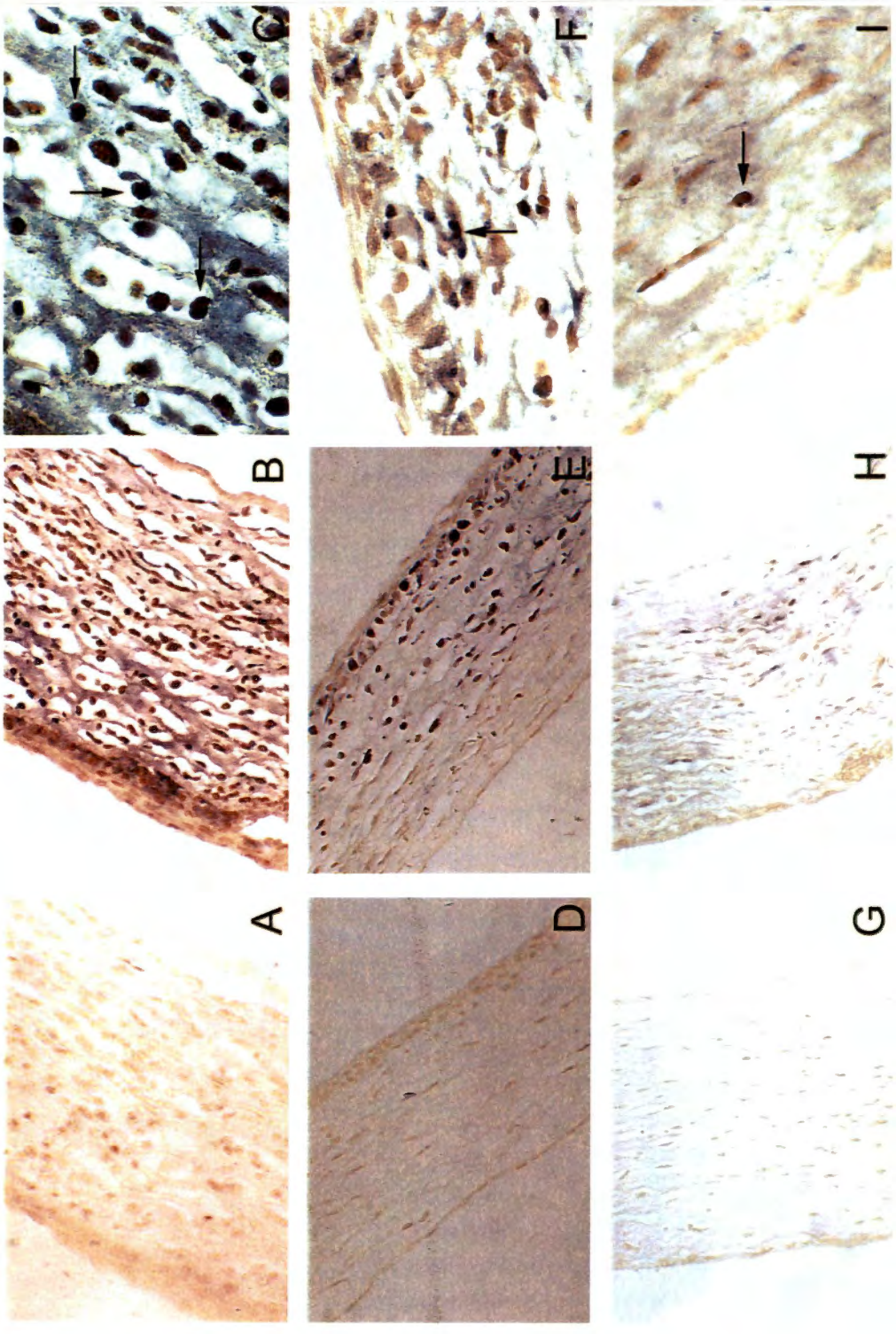
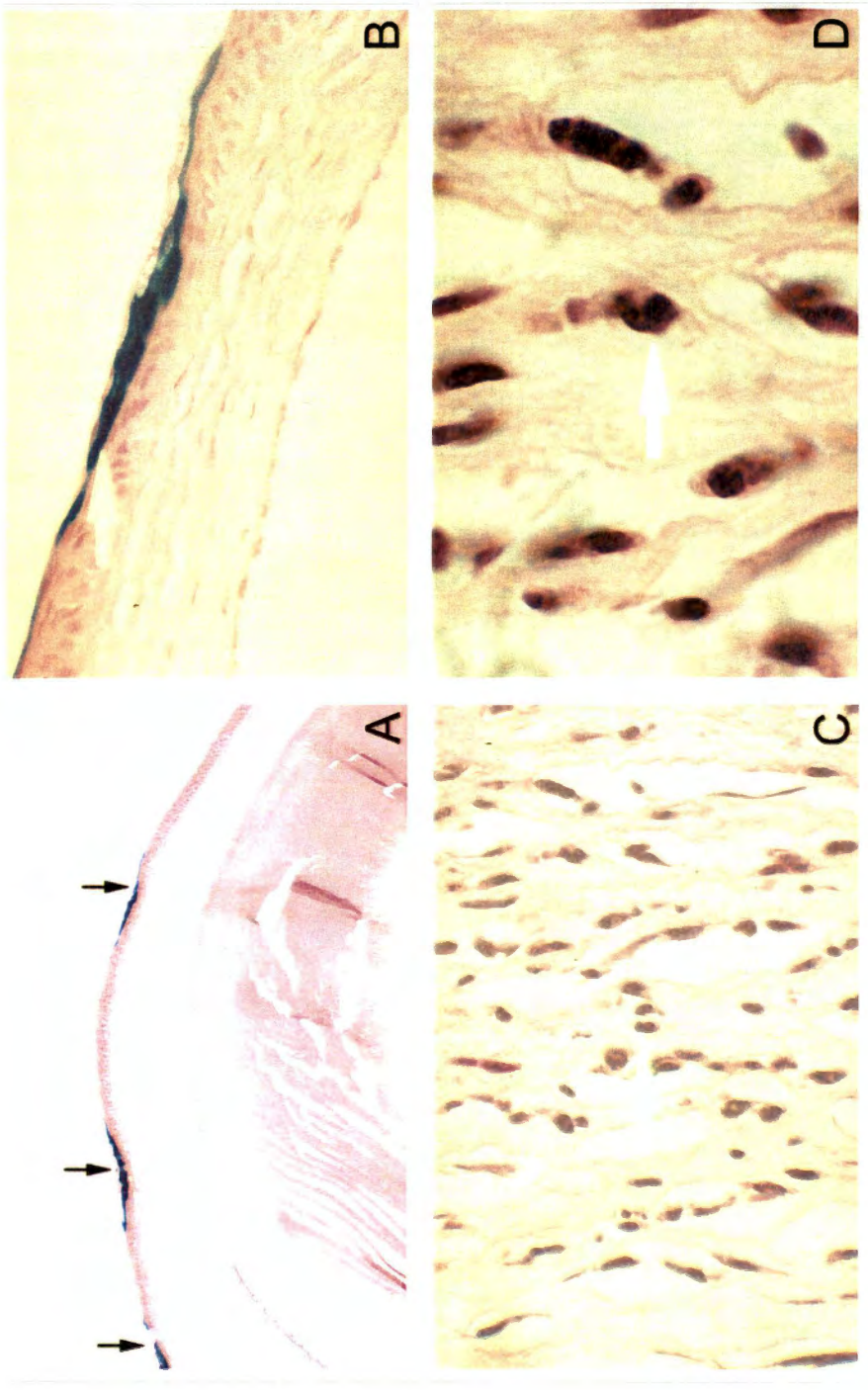

C
F
I
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Figure 2: **Localization of HSV in virus infected eyes.**

To locate virus in the infected eyes, HSV expressing B-gal was used. After infection (24 hrs) eyes were removed and stained for â-gal activity as described in Materials and Methods. Blue staining was located in epithelium layer shown by arrows (A, X62 and B, X250). To locate the inflammatory cells in the cornea upon HSV-1 infection, 24 h post-infected sections were stained with hematoxylin and eosin (C, D). Infiltrating cells were observed in stroma. The arrows show some infiltrating cells with polymorphic characteristics (C X250 and D X625).


B
D
A
C

PART III:

SUPPRESSION OF ON-GOING OCULAR INFLAMMATORY DISEASE BY TOPICAL ADMINISTRATION OF PLASMID DNA ENCODING IL-10

PURPOSE

Ocular infection with herpes simplex virus leads to an inflammatory lesion in the cornea orchestrated by CD4+ Th1 lymphocytes. This immunopathological disease, called herpetic stromal keratitis (HSK), is an important cause of impaired vision. These experiments were designed to determine if established lesions of HSK could be controlled by topically administering naked plasmid DNA encoding cytokines to the corneal surface. A single topical administration of DNA encoding IL-10 was beneficial to the majority (75%) of treated animals and 50% (vs. 10% in controls) resolved their lesions completely over a 23 day observation period. Topical ocular application of DNA encoding foreign proteins was also shown to be an effective means of inducing systemic and mucosal immune responses. The direct application of DNA encoding cytokines may represent an additional therapeutic option for the management of immunoinflammatory disease.

INTRODUCTION

Immune defense reactions against viruses often involve an immunoinflammatory response. This event is usually transitory and is a small tax to pay in most body locations for protection against cytodestructive viruses such as Herpes Simplex Virus (HSV). In the eye, however, the tariff is too costly since any inflammatory reaction along the visual axis impairs vision and its resolution may leave a scarred organ with impaired function [(1)]. Indeed as noted initially by Streilein and Kaplan, the eye appears to possess mechanisms which minimize inflammatory reactions [(2)]. Unfortunately these protective devices are not fully effective especially in the cornea. For example, ocular infection by HSV often

results in an inflammatory lesion called herpetic stromal keratitis (HSK) which is one of the commonest infectious causes of blindness in the USA. Indeed, of the many thousands of ocular infections with HSV which occur annually, 20% will result in some degree of HSK [3]. The human disease is suspected to be in large part immunopathological and experimental studies in the mouse model of HSK certainly confirm this viewpoint [1].

In the mouse, the mechanism of immunopathology involves T lymphocytes [1,4]. The crucial evidence that supports this notion is that the disease fails to occur if T cell function is lacking or abrogated and can only be reconstituted by adoptive transfer of T cells [4,5]. Most observations concur with the hypothesis that HSK is orchestrated by CD4+ T cells which produce cytokines of the type 1 profile [6,7]. Cytokines characteristic of Th1 cells dominate HSK in the preclinical and clinical phase, but in animals which undergo spontaneous resolution, Th2 cytokine expression, particularly IL-10, becomes evident [8]. In addition, HSK is minimized or abrogated if HSV infected animals are treated daily with ocular and systemic injections of IL-10 [9]. Taken together, these observations support the hypothesis that IL-10 is involved directly or indirectly in the resolution of HSK. In the present report, we have further evaluated the role of IL-10 in resolving lesions of HSK by measuring the effects of administering a single application of naked plasmid DNA encoding IL-10 to the cornea of animals expressing HSK. Although evidence accumulates to show that plasmid DNA encoding cytokines may prove useful to modulate immune induction and expression [10,11], to the authors' knowledge none have attempted to modulate such responses in the eye. In the present report, we have measured the consequences of exposure of the cornea of uninfected and HSK expressing mice to naked plasmid DNA encoding various proteins. Our data show prolonged expression in the eye

and the induction of both systemic and mucosal immune responses. More importantly, we demonstrate that treatment of eyes expressing HSK with naked DNA encoding IL-10 causes lesion remission. Our results add further evidence that IL-10 is mechanistically involved in HSK resolution and also indicate that the administration of naked DNA encoding cytokines may represent an additional means of treating an ocular disease that is an all too common cause of blindness.

MATERIALS AND METHODS

Mice : Four to five week old female BALB/c mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN) and acclimated for a week before use, in a specific-pathogen-free animal facility. Experimental procedures used in this study conformed to the resolution adopted by the Association for Research in Vision and Ophthalmology, on the use and care of laboratory animals.

Virus : HSV-1 strain RE and KOS were grown on monolayers of Vero cells maintained in DMEM containing 2% heat inactivated fetal calf serum (Life Technologies, Inc., Grand Island, NY) and titrated as previously described [5].

Plasmid DNA Preparation : Plasmid DNA encoding murine IL-10 (pcDSR-alpha mIL-10) containing the SV40 promoter and an internal ribosomal entrance site was a gift of T. Mossmann (Alberta, Canada) [12]. A control vector, termed pcSDR-alpha, was generated by excising the IL-10 gene by digestion with EcoRI, followed by ligation of the agarose-purified vector fragment. Plasmid DNA expressing murine GM-CSF (pRJB-GM) with the RSV LTR and murine IL-2 (pcDNAIII IL-2) containing CMV promoter were provided by H. Ertl (Philadelphia, USA)

[13] and plasmid controls for these constructs were respectively pRJB-c and PcDNA III. Plasmid DNA encoding glycoprotein B of HSV-1 (KOS) under the control of CMV promoter was described previously [14]. Plasmid DNA expressing B-galactosidase (PCMV beta) was purchased from Clontech (Cat. #6177-1). The vector control for the two last plasmids was Pc DNA I.

Plasmids were purified by polyethylene glycol (PEG) precipitation according to Sambrook et al. [15] with some modifications. Cellular RNA and proteins were precipitated with 1 volume of 7.5 M ammonium acetate followed by ispropanol precipitation of the supernatant. Residual cellular RNA was digested with 50 ug/ml of RNAse A at 37'C for 45 minutes. After PEG precipitation, plasmids were extracted with phenol : chloroform (3 times) and precipitated with 2 volumes of pure ethanol. The supercoiled conformation of the plasmid DNA was verified by electrophoresis on 1% agarose gel.

Corneal infection and clinical observation : Mice were deeply anesthetized with methoxyflurane (Metofane; Pittman-Moore, Mondelein, IL), and corneas were scarified using a 27 g half-inch needle in a criss-cross pattern. HSV-1 RE (1×10^6 $TCID_{50}$ in 4.0 ìl volume) was placed on the cornea and massaged with the eyelids. Animals were examined daily after infection by slit lamp biomicroscopy (Keeler Instruments, Broomag, PA) and the severity of the stromal keratitis in each cornea was graded from 0 to 5 as described elsewhere [8]. At the termination of the experiments, eyes were collected for histological evaluation.

Plasmid DNA Administration : In order to evaluate the therapeutic effects of plasmid DNA encoding different cytokines, HSV infected mice showing eye lesions with a clinical score of 2 at day 9 post infection, were

given various preparation of plasmid DNA. The mice were randomized in order to avoid personal bias in evaluating the efficacy of treatment. Plasmid DNA pellets were dissolved in DNase free sterile water to a concentration of 25 ug per ul. For the dose and kinetic experiments, varying concentrations of plasmid DNA, (4 ug, 10 ug, 25 ug, 100 ug), in 4.0 ul volume were applied to the scarified cornea of deeply anesthetized mice and gently massaged with the eyelids. For intramuscular (IM.) administration, a total of 200 ug of plasmid DNA was injected in the left quadriceps muscle and left and right biceps muscle. In each experiment half of the animals were exposed to test plasmid encoding cytokines and the other half were given the plasmid control without the respective cytokine genes.

B-galactosidase : At different time after intraocular administration of plasmid DNA encoding B-gal or vector control (PcDNA1), whole eye and cervical lymph nodes were removed and fixed in 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS) pH 7.2 for 1 hour at 4'C. Subsequently, the tissues were washed several times in PBS and placed overnight in substrate solution containing 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM $MgCl_2$, 2 mg of 5 bromo-4-chloro-3-indolyl-B-D galactoside (x gal) per ml, and 120 ul of 10% nonidet P-40 and 100 ul of 1% sodium deoxycholate per 20 ml. After the reaction, the whole tissues were washed in PBS, fixed in 3.7% formaldehyde. Tissues were embedded in paraffin and sectioned at 5 um and stained with nuclear fast red.

Histology : Eyes from traumatized mice, and from HSV infected mice (untreated, no IL-10 mice, mice given plasmid DNA encoding cytokines as well as mice that received vector control plasmid preparations) were removed and placed in 10% buffered neutral formalin, embedded in

paraffin, sectioned at 6 uM and stained with hematoxylin and eosin (H&E).

Isolation of RNA : Pooled corneas were minced into small pieces and treated with TRI-REAGENT (Molecular Research Center, Cincinnati, OH). The total RNA was then extracted with chloroform and precipitated with isopropyl alcohol. The contaminating plasmid DNA was digested by treating the RNA preparation with RQ-1 RNAse free DNAse (Promega, Madison, WI). The absence of plasmid DNA in the RNA preparation was verified by performing a polymerase chain reaction on the total cellular RNA without reverse transcription, using specific primers. The RNA obtained from 6 corneas, after DNAse treatment, was taken up in 40 ul of nuclease free water.

Reverse Transcription (RT) : Total cellular RNA (10 ul) was reverse transcribed in a 40 ul volume using oligo dT primers and reverse transcriptase (Superscript, Life Technologies, Inc., MD) according to the protocol described earlier [16]. The RT reaction was carried out at 42'C for a period of 90 minutes.

Polymerase Chain Reaction (PCR) : The cDNA was diluted 1:3 in sterile distilled water and subjected to 30 cycles of amplification (Denaturation at 94'C for 90 sec., annealing at 55'C for 60 sec., and extension at 72'C for 120 sec.) using specific sets of primers. The details of the conditions of PCR have been described previously in detail [16].

Estimation of IL-10 mRNA in the Cornea : Messenger RNAs were reverse transcribed using Oligo dT primers and superscript reverse transcriptase (Life Technologies Inc., MD). The level of IL-10 mRNA was estimated using a quantitative competitive PCR employing a multi-specific competitor, as described previously [16]. Briefly, equal amounts of

cDNA were mixed with varying concentrations (known amounts) of the competitor and were amplified simultaneously using primers specific for IL-10. The PCR products were analyzed on a 2% agarose gel, stained with ethidium bromide and photographed. The intensities of the competitor and target bands were measured using densitometric scanning (Model GS-670 Imaging densitometer, Bio-Rad, Richmond, CA). Using the values of the competitor, the concentration of target IL-10 was calculated as described previously [16].

Ribonuclease Protection Assay : Total cellular RNA (25 ug) obtained from corneas were hybridized with ^{32}P labeled mRNA probes generated from template mCK 2 (Pharmingen, San Diego, CA). In addition to IL-10, the mixture of labeled probe also contained anti-sense mRNAs for IL-12, (p35, p40) IL-6, IL-1, INF-alpha, MIF and GAPDH. The hybridization reaction was carried in 20 ul volumes at 56'C for a period of 16 hours. The unprotected RNAs were digested using a mixture of Ribonuclease A and T1. The protected RNAs were isolated from the mixture using phenol-chloroform extraction followed by ethanol precipitation. The hybridized labeled RNAs were analyzed on a 6% polyacrylamide gel (containing urea), desiccated and exposed to X-Ray film (Cronex MRF-34, Dupont). The probe preparation, purification, and hybridization were all carried out according to the protocol discussed in detail elsewhere [17].

ELISA : Serum was collected by retroorbital bleeding. Vaginal washings were collected with a micropipette by introducing 100 ul of PBS into the vaginal cavity, and 50-70 ul were recovered per animal. The vaginal lavage sediments were subsequently removed by centrifugation, and 50 ul,diluted four times in PBS, volumes of each individual sample were analyzed. Ocular lavages were collected by washing corneal surfaces of the individual mice(20 ul of PBS per mouse). The ocular lavages from ten

mice were pooled for detection of gB-specific IgG or IgA. Fecal samples were weighed and suspended in PBS containing 0.1% sodium azide at a concentration of 100 mg/ml. Samples were shaked vigourously for one hour and centrifuged for 15 min. to collect the supernatant. For detection of Ab against gB, enzyme-linked immunosorbent assy (ELISA) plates were coated with gB protein at a concentration of 2 ug/ml. A standard ELISA was performed using goat anti-mouse IgA, IgG, IgG1 or IgG2a to determine the gB -specific Ab in the samples and the concentration of Ab was determined from the standard curve using known amounts of mouse IgA, IgG, IgG1 or IgG2a. Briefly, after overnight incubation at 4'C with gB protein or anti-mouse IgG, the plates were washed three times with PBS containing 0.05% Tween 20, pH 7.2, (PBST), and blocked by using PBS pH 7.2 with 3% dehydrated milk for two hours at 37'C. A total of 200 ul of serum samples (prediluted in PBST) were added in duplicate and serially diluted two fold to a final 100 ul final volume. Wells coated (100 ul) with 2 ug per ml of goat anti-mouse IgG or IgA were treated with serial diluted standard mouse IgA, mouse IgG, mouse IgG2a, or IgG1. Plates were incubated for two hours at 37'C. After being washed, 100 ul of goat anti-mouse IgG-conjugated horse radish peroxidase (IgGHRP) were added and wells were incubated for one hour. For detection of IgG isotypes, goat anti-mouse IgGHRP or goat anti-mouse IgG2aHRP was added. After three washes, 2,2-azino-bis-3-ethylbenz-thiazoline-6-sulfonic acid substrate was added (22 mg of substrate plus 20 ul of 30% H_2O_2 + 50 ml of 0.1 M of citric acid + 50 ml of 0.1 M Na_2HPO_4). Antibody concentrations were determined from the standard curve. Mouse serum responses were measured individually. The standard deviation of the serum concentrations were based on individual serum samples from 10 mice per group.

Cytokine Quantitation in Cornea : Three days after administration of 100 ug of IL-10 plasmid, IL-2 plasmid, and their respective plasmid controls, the corneas were collected, excised free of scleral tissues, and transferred to DMEM (Gibco). The samples were frozen at -80'C, thawed at 42'C, homogenized for in 45s (Pro Scientific Inc. Pro 0200, Monroe, CT, USA) and centrifuged 1 min. at 10000 g at 4'C. The supernatants were analyzed for IL-10 and IL-2 production by ELISA. ELISA plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight at 4'C with 2 ug of rat anti mouse IL-2 or IL-10 and IL-10 (Pharmingen Cat #180000 for IL-2; Cat #181410 for IL-10). Plates were subsequently blocked with 3% skim milk for 1 hour 37'C. The samples, and recombinant IL-10 or recombinant IL-2 (Pharmingen, San Diego, CA, USA, Cat #19281V, Cat #19291V, respectively) at concentration 100 ng/ml were added to the plates and serially diluted. The standards and the samples were incubated overnight at 4'C. After washing, 100 ìl of biotinylated anti IL-10, or IL-2 antibodies (Pharmingen Cat #18172D for IL-2, Cat #18152D for IL-10) at a concentration 1 ug/ml were added and the plates were incubated at 37'C for 1 hour. After washing, streptavidin HRP conjugated (Jackson ImmunoResearch, West Grove, PA, USA, Code 016-030-084) was added. The ELISA was developed as previously described [18].

HSV-specific Lymphoproliferation Assay : To determine if the ocular gB DNA immunization could induce an HSV-1 specific lymphoproliferative response, 100 ug gB DNA was administered on scarified eyes of BALB/c mice on day 0 and day 10 . Same amount of control DNA (pc DNA 1 without gB) or 10^6 $TCID_{50}$ of HSV-1 KOS (live) were controls. On day 30, immune splenocytes (responders) were collected and restimulated in vitro with HSV-1 KOS (moi of 1.5 before

u.v. irradiation). Lymphoproliferation was measured as described previously [18].

Quantification of Cytokine Producing Cells After In-vitro Restimulation : Fifteen days after the last intraocular gB DNA, vector plasmid as HSV immunization, the animals (3 per group) were sacrificed and the spleens were aseptically removed. Single cell suspensions were made from the spleens and subsequently treated with ammonium chloride-containing Tris buffer for 3 min. at 37'C to deplete the erythrocytes. These cells were used as responders. Enriched dendritic cell (DC) populations were used as stimulators. Briefly, splenocytes from naive female retired breeders were depleted from erythrocytes and 3 ml of 10^7 cells were layered over 2 ml of metrizamide gradient (Nycomed Pharma A, Oslo, Norway; analytical grade, 14.5g added to 100 ml PBS, pH 7.0). The cells were then centrifuged at 600 g for 10 min. and the cell interface was collected. The enriched DC population was pulsed for 3 h with uv inactivated (UV-HSV) HSV at a multiplicity of infection of 5 (prior to inactivation). Subsequently, the cells were washed and counted. The responder cells and the DC (naive or pulsed with uv HSV) were added at a responder to stimulator ratio of 50:1, 25:1, 12.5:1, 6.25:1 in 200 ul RPMI 10% FBS per well into the coated and blocked ELISPOT plates. ELISPOT plates were precoated with anti cytokine antibodies (PharMingen anti IL-5 cat. #18051D, anti IL-4 cat. #18031D, anti IFN-alpha cat. #18111D) at concentration 2 ug/ml in 100 ul in sterile carbonate buffer at 4'C overnight and were subsequently blocked with RPMI 10% FBS media for 1h at 37'C. After 72h incubation of the responder and stimulator cells (in a vibration free incubator), the ELISPOT plates were washed with PBS (3x) and PBS-Tween (3x) and 100 ul of biotinylated anti cytokine antibodies (Pharmingen, biotinylated anti IL5 cat. #18062D,

biotinylated IL-4 cat. #18042D, biotinylated INF-alpha cat.#18112D) diluted 1:1000 in PBS-Tween 1% FBS were added to the plates. After 1h incubation at 37'C, 100 ul of 1:1000 diluted alkaline phosphatase-conjugated streptavidin in PBS TW20 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. Cells from animals that received plasmid DNA (vector control) or, 1 x 10^6 PFU of HSV-1 KOS ocularly were used as negative and positive controls, respectively.

Delayed-type Hypersensitivity (DTH) : Test antigens were injected in 20 ul volumes in the ear pinna, and ear thickness was measured with a screw gauge meter 48h later as described in detail elsewhere [19]. Test antigens included were u.v. inactivated HSV-1 (KOS) with a titer of 1 x 10^5 (prior to u.v. inactivation). Vero cell extract injected in the left ear pinna was used as the negative control. Results were expressed as mean increase in ear thickness at 48 h following ear pinna injection over the prechallenge thickness. Five mice per group were used.

RESULTS

Ocular Gene Expression : Following topical administration of plasmid DNA encoding various genes to the scarified cornea of anaesthetized mice, evidence of gene expression was measured both in the eye as well as in the draining lymph node (DLN). The expression occurred was measured by several approaches. Firstly, as shown in Fig. 1a, using quantitative PCR to measure levels of IL-10 mRNA in pooled corneal samples, significant signals were detectable at least until 11 days post exposure. Maximal responses were noted in mice exposed to 100 ug DNA but above background signals were observed in animals exposed to 10 fold less DNA

(Fig. 1b). Further confirmation for IL-10 gene expression in the cornea as well as the DLN was afforded by a ribonuclease protection assay (Fig. 1c), as well as by measuring IL-10 protein by ELISA in homogenized corneal samples (Fig. 1d). In the latter experiments, control mice received either vector or plasmid DNA encoding IL-2. In samples from the latter group, IL-2, but not IL-10, was readily detectable.

In an attempt to determine the site of gene expression, the scarified corneas of naive and animals with HSK, were exposed to plasmid DNA encoding B-galactosidase. Such treatment led to B-gal mRNA expression in both corneas and DLN as detected by semiquantitative RT-PCR (data not shown). To localize B-gal protein expressing cells, paraformaldehyde fixed tissue was reacted with X-gal and wax embedded tissues were evaluated histologically. Occasional positive cells were evident in the epithelium and stroma of naive animals examined at 30 hour post exposure (Fig. 2a, b). Abundant signal was detectable in the iris and ciliary body particularly in 3 animals examined at 6 days post-application (Fig. 2c, d). No positively staining cells were observed in the retina. Isolated positive cells were also observed in DLN at least until 6 days post exposure (data not shown).

Animals with mild HSK lesions were also exposed via the scarified cornea to plasmid DNA encoding B-gal. Again the predominant signal was observed in the iris, but more positive cells were present in the stroma than was noted in the same tissue of naive animals (Fig 2e, f). The identity of cells expressing B-gal was not established.

Finally evidence for gene expression following corneal exposure to plasmid DNA was provided by measuring immune responses in animals exposed to plasmid DNA encoding the HSV major glycoprotein gB. For

this purpose, sera, vaginal washes, fecal samples, and splenic lymphocytes were collected 15 days after a second ocular administration of gB DNA. As shown in Table 1, gB specific IgG was detectable in serum and gB specific IgA was present in feces and vaginal wash samples. Levels were lower than in control animals immunized with avirulent HSV, but the mucosal responses were comparable to those achieved following intranasal immunization with gB DNA (data not shown). Cell mediated immune responses to gB were detected in several ways. Firstly, animals exposed topically to gB DNA demonstrated positive skin tests when tested for delayed type hypersensitivity to HSV (Table 1). In addition, splenocytes tested in vitro for HSV antigen specific reactivity by lymphoproliferation as well as by the ELISPOT approach to enumerate antigen specific cytokine producing cells showed positive responses in animals topically exposed to gB DNA. The results of ELISPOT analysis revealed positive responses for IFN-alpha producing cells but insignificant responses in terms of IL-4 and IL5 SFC. Accordingly, the nature of T cell immunity following ocular exposure to DNA appears as Th1 type just as occurs after intramuscular immunization with DNA [18].

Modulation of HSK With Cytokine DNA : As documented previously [8], HSK first becomes clinically evident in HSV infected BALB/c mice at 8-10 days post infection. To measure the possible modulatory effects of cytokine DNA, mice were selected that were showing early 2+ lesions as measured by slit lamp biomicroscopy. Plasmid DNA was applied on a single occasion to the scarified corneas of anaesthetized mice and the progress of clinical expression was followed in individually labeled mice over an ensuing three week observation period. In each experiment, animals either received cytokine DNA or control plasmid DNA. The results of various experiments are shown in Fig. 3. As is clearly evident,

the majority of animals treated with IL-10 showed lesions of diminished severity in comparison to control plasmid treated individuals where lesions usually increased in severity (Fig. 3a).

In the IL-10 treated group, approximately 75% of eyes showed controlled or resolved lesions during a 23 day observation period. Moreover, those still showing non-advancing lesions resolved them completely 50 days post treatment. In control plasmid treated eyes resolution was evident in a maximum of 15% of eyes. Histological sections of corneas from animals treated with IL-10 DNA and showing lesion remission clinically (Fig. 4a) revealed a cornea virtually indistinguishable in tissue structure to that of naive uninfected animals (Fig. 4b). By way of contrast the large majority of corneas from animals given the control vectors showed lesions (Fig. 4c) advanced well beyond lesion severity at the start of experiments (Fig. 4d).

The pattern of events observed following topical ocular treatment with IL-10 DNA failed to occur in animals similarly treated with IL-2 DNA (Fig. 3b), GM-CSF DNA (data not shown) or in animals given a single intramuscular administration of IL-10 DNA (Fig. 3c). Accordingly, our results indicate that for IL-10 to function and cause HSK resolution cytokine expression is likely required in ocular tissues.

DISCUSSION

This report demonstrated that the application of naked plasmid DNA encoding various proteins to the scarified cornea lead to prolonged gene expression at the site and dissemination to the draining lymph nodes. Gene expression was demonstrated in several ways including the detection of mRNA and protein in corneal extracts and the induction of systemic and

mucosal immunity in animals exposed to plasmid DNA encoding the HSV glycoprotein gB. Most notably, it was observed that ocular administration of DNA encoding the cytokine IL-10 was a valuable means of modulating the expression of an ocular immunoinflammatory disease induced by HSV infection. This observation, that expression of plasmid DNA regularly occurred following topical application to a mildly abraded corneal surface was unexpected. Lesser and more irregular expression occurred following application to the undamaged cornea (data not shown) and this approach, if combined with a more effective delivery system such as liposome entrapment, might provide a practical means of achieving gene transfer and immunization. Ocular delivery led to detectable gene expression for almost two weeks and animals developed systemic immunity as well as specific IgA responses at distal mucosal sites when given DNA encoding foreign proteins. The systemic response presumably resulted from transfer of plasmid to the DLN and expression at this site was readily demonstrable. In fact, it was common to observe DLN enlargement following topical ocular application of plasmid DNA encoding foreign proteins such as gB and B-galactosidase. The induction site for the mucosal IgA response remains to be shown. Conceivably some plasmid gained access to the nasal mucosa where it was shown previously that naked plasmid DNA induces distal mucosal responses in the vaginal and alimentary tracts [13]. The extent of gene expression in various lymphoid tissues following topical application of plasmid DNA encoding foreign proteins is under investigation. Although currently the main focus on plasmid DNA is its' possible utilization as a means of vaccination, an alternative use may be to achieve immunomodulation. These results add clear support for this possibility. Accordingly, the majority of animals expressing an immunoinflammatory reaction in the cornea as a

consequence of HSV infection resolved or contained their lesions upon a single topical application of plasmid DNA encoding the cytokine IL-10. Similar effects were not achieved with DNA encoding IL-2 and GM-CSF, the only other cytokine DNAs tested so far. The reason for the focus on IL-10 was because HSK is assumed to represent an immunopathological disease orchestrated by Th1 CD4+ T cells, and IL-10 is known to counteract the induction and some of the functions of Th1 producing CD4+ T cells [14,15]. In addition, IL-10 may inhibit some of the properties of neutrophils [16], prominent cell types in HSK lesions [17]. That IL-10 may be involved in HSK resolution, was suspected following the observation that IL-10 gene expression is upregulated in corneas which spontaneously resolve their lesions [8]. Moreover, the daily administration of IL-10 protein systemically and into the corneal stroma may modulate HSK, but this does not represent a practical approach for immunotherapy. Our observation, which appears to be novel, may provide a practical means of modulating a disease which in humans is a significant cause of vision impairment and frequently necessitates corneal transplantation. Although our results clearly show the benefits of IL-10 DNA therapy, it is left to be shown how the approach functions mechanistically and if DNAs encoding other cytokines might complement or act even more effectively than IL-10. It is also likely that the immunomodulatory effects of cytokines or immunoinflammatory disease may be enhanced by the use of delivery systems which achieve more effective penetration into inflammatory tissue. There is current evaluation of several delivery systems for this purpose going on. Also, there is work attempting to determine whether IL-10 expression in some particular cell type in the eye accounts for the effects observed.

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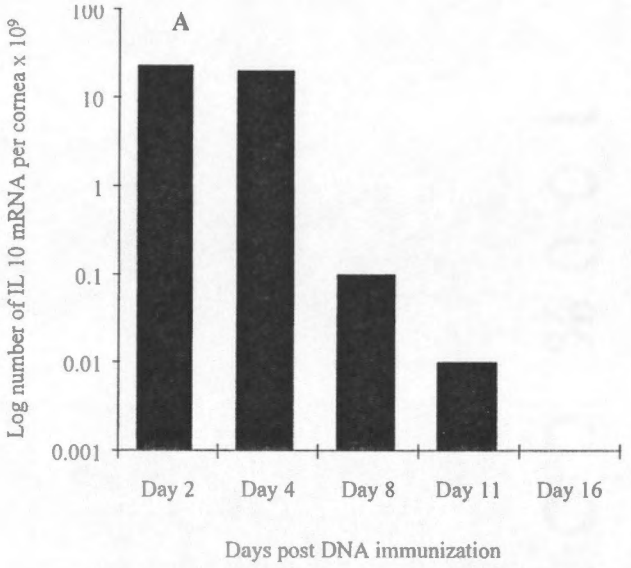
Figure 1: **Expression of IL-10 mRNA and protein in the cornea.**

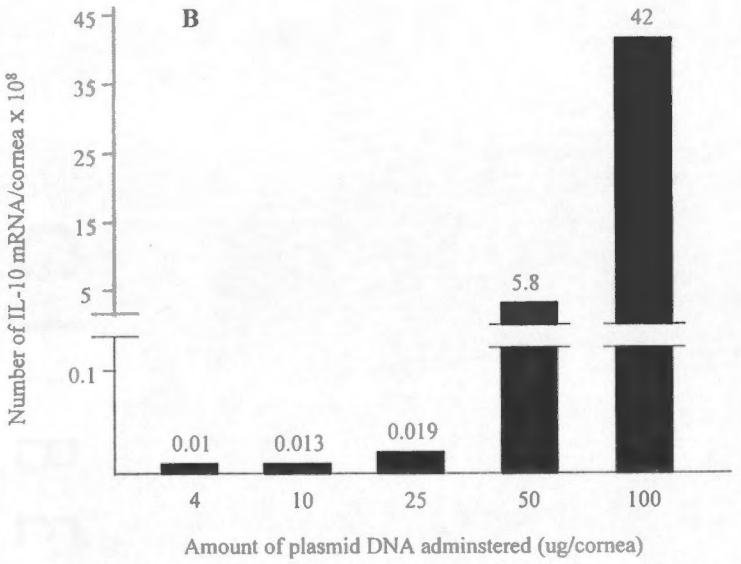
A. Scarified corneas (n = 6) were exposed to 100 ug of plasmid DNA encoding IL-10. Corneas were collected on days 2, 4, 8, 11, and 16 following DNA administration. Total RNA was isolated from corneas, reverse transcribed and the levels of IL-10 mRNA per corneas were estimated by quantitative competitive PCR (methods).

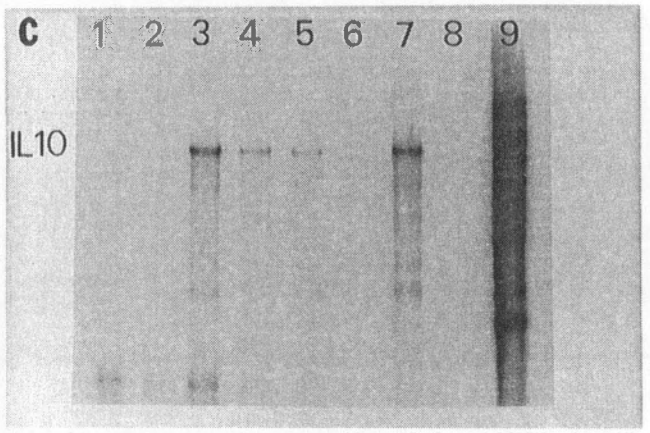
B. Scarified corneas were exposed to varying amounts of plasmid DNA-IL-10 (4.0 ug, 10.0 ug, 25.0 ug, 50 ug, and 100 ug). Corneas were collected on day 4 following DNA administration. The amount of IL-10 per cornea was measured by quantitative competitive PCR.

C. Demonstration of IL-10 mRNA in the cornea and DLN by ribonuclease protection assay: Total RNA, obtained from corneas and DLN on day 4 following plasmid DNA-IL-10 administration (100 ug/eye), was hybridized with a mixture of ^{32}P labeled antisense mRNA probes. The mixture of probes, in addition to IL-10, contained IL-12 (p40, p35), IL-1, IL-6, TNF, and IFN-gamma. The unhybridized (unprotected) RNA was digested using a mixture of RNAse T_1 and A. The protected (hybridized) mRNAs were resolved on a 6% polyacrylamide-urea gel, dissected and exposed to X-ray film, and photographed. Lanes : 1. cornea: vector control. 2. DLN: vector control. 3. cornea: IL-10 plasmid (pooled n = 20). 4. DLN: IL-10 plasmid. 5. cornea: IL-10 plasmids (pooled n = 10). 6. negative control: IL-10 antisense RNA. 7. positive control: IL-10 sense RNA. 8. probe treated with RNAse. 9. probe mixture.

D. Demonstration of IL-10 protein in the cornea. Three days after administration of 100 ug of IL-10 plasmid, IL-2 plasmid and their respective vector controls, corneas were collected, washed in DMEM and were frozen in DMEM at -80'C. The corneas were then thawed at 42'C and homogenized for 45 seconds. The lysate was clarified by centrifuging for 1 minute at 10,000 g at 4'C. The supernatants were analyzed for IL-10 and IL-2 protein using ELISA (methods).


A
Log number of IL 10 mRNA per cornea x 10⁹
100
10
1
0.1
0.01
0.001
Day 2
Day 4
Day 8
Day 11
Day 16
Days post DNA immunization


B
Number of IL-10 mRNA/cornea x 10⁸
45
35
25
15
5
0.1
0.01
0.013
0.019
5.8
42
4
10
25
50
100
Amount of plasmid DNA adminstered (ug/cornea)


C
1 2 3 4 5 6 7 8 9
IL10

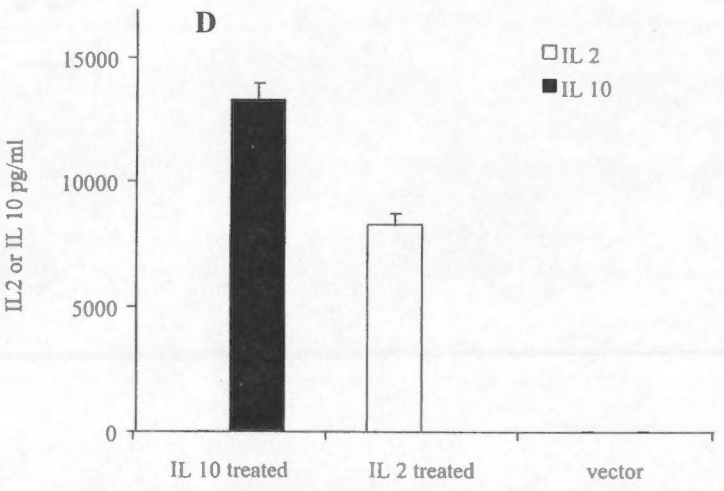

D
IL 2
IL 10
IL2 or IL 10 pg/ml
15000
10000
5000
0
IL 10 treated
IL 2 treated
vector

Figure 2: **Expression of B-galactosidase in the eye.**

Plasmid DNA encoding B-galactosidase was administered to the scarified cornea, and the eyes were processed at different times, as described in methods, for the location of B-galactosidase expression.

A&B. Naive eye 30 hr post treatment showing signal in the epithelium (A) (x125) and stroma (B) (x500)

C&D. Naive eye 6 days post treatment showing signal in the iris (C) (x125) and ciliary body (D) (x125)

E&F. The eye of animal with 2+ HSK 30 hr after topical treatment with B-gal DNA showing signal in epithelium, stroma, iris and ciliary body (A) (x125) and stroma only (B) (x250)

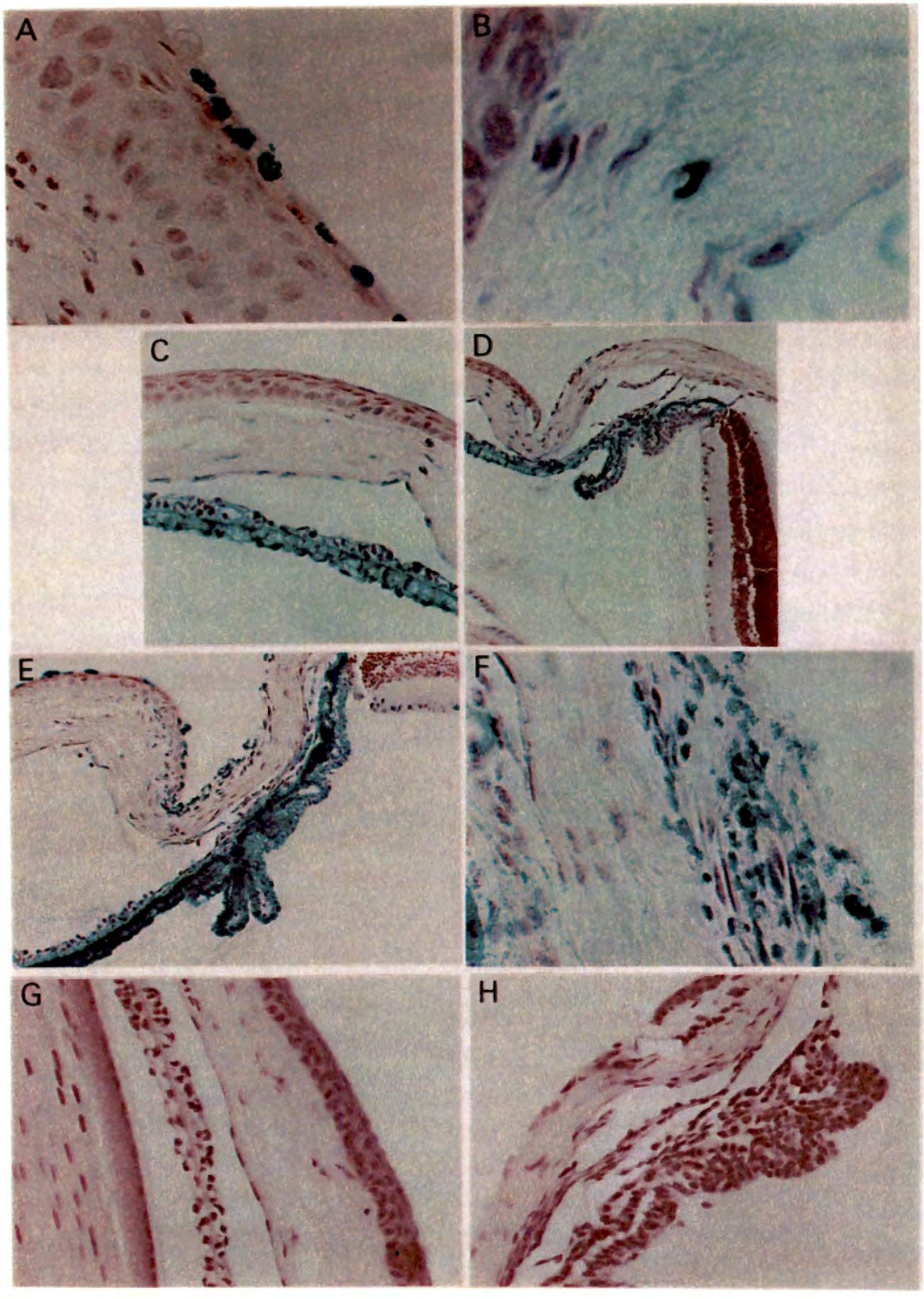

A
B
C
D
E
F
G
H

Figure 3: **Course of clinical lesions in treated and control animals.**

A. Groups of animals with a score of 2+ clinical lesions were given 100 ug each of IL-10 plasmid or IL-2 plasmid or respective vector controls intraocularly. The animals were then examined for the course of clinical lesion by slit lamp biomicroscopy examination. Scores of individual eyes are shown on the graph. Fig. 3A records results of a single experiment involving 20 eyes treated with IL-10 DNA and 20 given control plasmid. In three other experiments of a limited design involving a total of 52 IL-10 treated and 52 control plasmid treated eyes 79% of animals treated with IL-10 showed reduced or completely resolved lesions. This compares with 11% in the control plasmid treated group.

B. This figure shows an experiment of the same design comparing IL-2 DNA treated eye with control plasmid treated eyes.

C. This figure shows the results of an experiment in which animals were given IL-10 DNA intramuscularly on a single occasion.

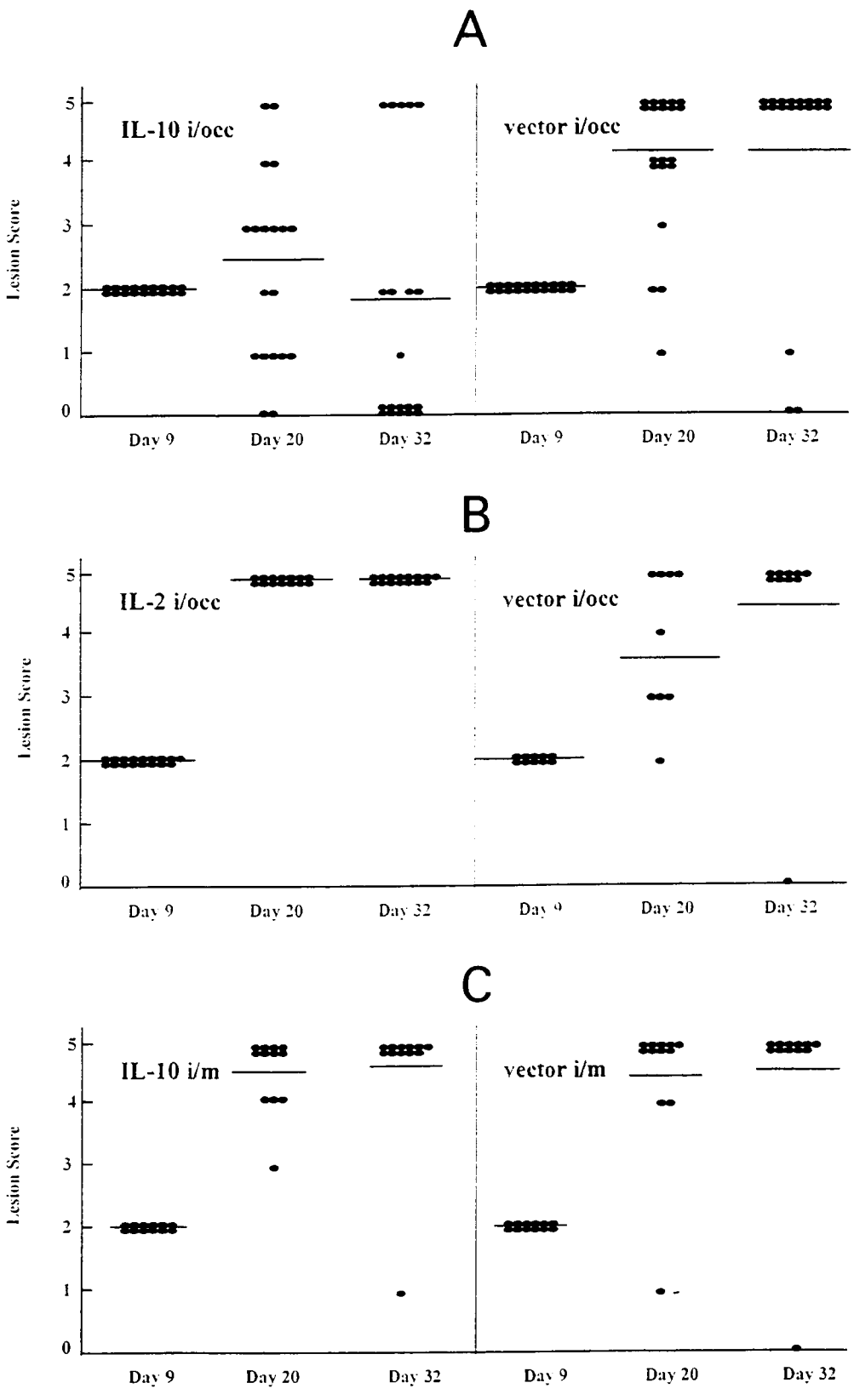

A
IL-10 i/occ
vector i/occ
Lesion Score
0
1
2
3
4
5
Day 9
Day 20
Day 32
B
IL-2 i/occ
vector i/occ
C
IL-10 i/m
vector i/m

Figure 4: **Histological sections of eyes stained with H&E obtained from treated and untreated animals (x125).**

A. Section from HSV infected eye 23 days post-treatment with IL-10 DNA.

B. Section from naive, uninfected eye.

C. Section from HSV infected eye 23 days post treatment with control plasmid DNA.

D. Section of HSV infected eye at the time of treatment with plasmid DNA.

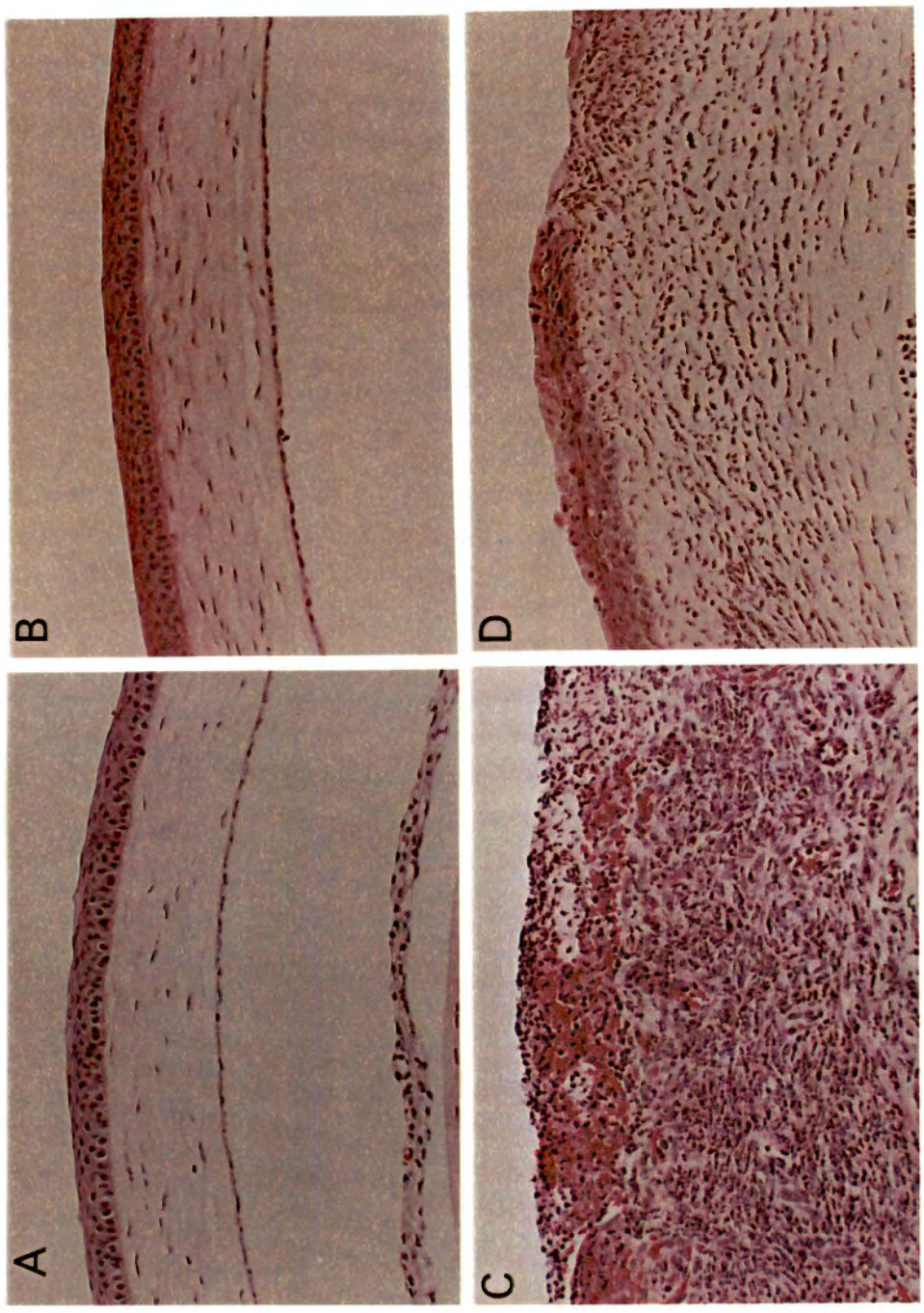

A
B
C
D

Table 1. **Immune Responses to ocular gB DNA or live HSV administration**

Parameter	gB I/OCC	vector I/OCC	HSV-1 I/OCC
Serum IgG[1]	79 ± 52	<5	5380 ± 200
Fecal IgA	20 ± 6	0	369 ± 40
Vaginal IgA	16 ± 5	<5	831 ± 74
IFNgamma SFC[2]	152 ± 29	23 ± 3	590 ± 70
IL-4 SFC	10 ± 4	4 ± 2	69 ± 10
IL-5 SFC	3 ± 2	0	48 ± 8
Lymphoproliferation[3]	8.3	<1	11
DTH[4]	5.4 ± 2.6[a]	1.2 ± 0.8[b]	10.8 ± 3.7[c]

[1]Serum, fecal and vaginal samples from BALB/c mice given gB DNA, vector DNA or live HSV intraocularly three times were tested for anti-gB antibody responses by ELISA. Samples were collected fifteen days following the last immunization and tested individually. Each group consists of 7 to 11 mice. The values are presented as [ng/ml].

[2]Fifteen days following the last immunization, splenocytes from the immunized mice were in vitro restimulated for 3 days with naive or UV-HSV pulsed dendritic cell (DC) populations. The number of SFC (spot forming cells) was determined by ELISPOT assay. The number of cytokine SFC after naive DC restimulation was subtracted from the number of SFC obtained following HSV-pulsed DC restimulation.

[3]HSV-specific proliferation assay. Immune responder cells were mixed with irradiated syngeneic spleen cells infected with 1.5 moi UV-HSV-1 (stimulator cells) and incubated at 37'C for 5 days as described in Methods. The ratio between the cpm of responders plus HSV infected stimulators and responders plus naive stimulator were calculated and expressed as stimulation index. Data represents one of the 3 experiments performed. Each showed similar results.

Table 1. cont.

[4]DTH analysis of BALB/c mice ocularly immunized with plasmid DNA or live HSV-1 (KOS) as described in methods. Data present the mean difference (10^{-2} mm) between the thickness of left and right ear pinnae in each group at 48 h post challenge (a vs. b $P = 0.008$; a vs. c $P = 0.02$; b vs. c $P < 0.001$).

PART IV:

IMMUNE INDUCTION AND MODULATION BY TOPICAL OCULAR ADMINISTRATION OF PLASMID DNA ENCODING ANTIGENS AND CYTOKINES

PURPOSE

In this report, the potential for administration of a eukaryotic expression vector, and its' effect in the modulation of the ocular inflammatory process were investigated. Plasmid DNA delivered to the ocular surface via scarification or drop method provided a means of inducing and modulating the immune response to herpes simplex virus (HSV-1). Topical application of gB DNA led to the development of HSV specific systemic humoral and cellular immunity as well. In addition, mucosal antibody was induced at both proximal and distal locations. Topically gB DNA immunized animals were protected against lethal challenge with HSV-1 via either the systemic or the vaginal mucosal routes. Ocular pre-exposure to DNA encoding the cytokines IL-4 or IL-10, but not IL-2 or interferon gamma, modulated the severity of the immunoinflammatory response to subsequent corneal infection with HSV. These results indicated that the ocular surface provides a readily accessible site for DNA immunization and was suitable for both immune induction and modulation of the nature of the immune response to the pathogen.

INTRODUCTION

Although initially surprising to most investigators, the observation that bacterial plasmid DNA encoding genes for various proteins induced immune responses upon intramuscular injection, is now widely accepted. Indeed DNA vaccination has moved from conception to clinical trials in a short 5 year period and some infections agents currently lacking effective vaccines might be controlled by the DNA approach [1,2]. The favored means of attaining DNA-induced immunity is by either intramuscular injection or by gene gun administration into the skin [3]. It became evident

recently that intranasal administration of DNA also induced immunity, and this approach was more effective than the IM route in inducing mucosal immunity, an important aspect in the prevention of pathogen invasion [4]. DNA immunization has potential applications beyond the prophylaxis of infectious disease. For example, DNA immunization promises to be a valuable means of achieving immunomodulation by administering DNA-encoding specific cytokines along with antigen encoding plasmids [5,6]. Conceivably the approach has value to change the pathological nature of immune responses in allergy, cancer, and autoimmunity [7,8,9].

Of particular interest, DNA immunization with plasmids encoding cytokines represents a valuable method of modulating the severity of on-going immunoinflammatory disease [5,6]. Thus we showed that topically administered DNA encoding the cytokine IL-10 caused remission of ocular immunoinflammatory herpetic lesions [5]. The observation that topical application, albeit to the scarified cornea, led to gene expression of the plasmid-encoded gene of interest came as a surprise. In the present communication we have further explored the value of the eye as a means of inducing protective immunity and providing immunomodulation by administration of eukaryotic expression plasmids. Our data showed that ocular administration of DNA encoding a glycoprotein gene of herpes simplex virus (HSV), successfully induces systemic as well as mucosal immunity. In addition, we show that prophylactic administration of various cytokine DNAs to the cornea provide a reliable means of tailoring the nature of the subsequent immunoinflammatory response to ocular infection with HSV virus.

MATERIALS AND METHODS

Mice : Age-matched four to five-week old female BALB/c mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and housed for 1 week before use in a specific pathogen-free animal facility. Experimental procedures used in this study conformed to the resolution adopted by the Association for Research in Vision and Ophthalmology, on The Use and Care of Laboratory Animals.

Virus : HSV-1 strain RE, McKrae, 17 and KOS were grown on monolayers of vero cells maintained in DMEM containing 2% heat-inactivated fetal calf serum (Life Technologies, Grand Island, NY) and virus was titrated, as described previously [10].

Cutaneous and Vaginal Challenge : DNA or HSV-1 (KOS) immunized mice were challenged cutaneously with 10^5 (100 50% lethal doses [100 LD_{50}]) pfu of HSV-1.17 using the mouse zosteriform model as described previously [11]. For the vaginal challenge, the estrus cycle of immunized mice was synchronized at the progesterone dominant stage with Depo-Provera (DP) (Upjohn Company, Kalamazoo, MI) [12]. Mice were injected subcutaneously with 2 mg DP in 50 ul of distilled water. Five days after the administration of DP, the mice were challenged intravaginally with 2 x 10^5 (100 LD_{50}) pfu of HSV-1 McKrae.

Corneal Infection and Clinical Evaluation : Mice were anesthetized with methoxyflurane (Metofane; Pittman-Moore, Mondelein, IL), and corneas were scarified with a 27 g half-inch needle in a criss-cross pattern. A 4 ul drop containing 10^6 $TCID_{50}$ HSV-1 RE virus particles was placed on the cornea and massaged into the cornea with the eye lids. Animals were examined daily after instillation by slit lamp biomicroscopy (Keeler Instruments, Broomage, PA), and the severity of the stromal keratitis in

each cornea was graded from 0 to 5, as described elsewhere [13]. At the termination of the experiments, eyes were collected for histologic evaluation.

Plasmid DNA Preparation : All the plasmids were purified by polyethylene glycol precipitation according to Sambrook et al. [14] with some modifications as described previously [4]. Plasmid DNA encoding gB of HSV-1 (KOS) with CMV promoter was described previously [15]. Plasmid encoding cytokine IL-2 with the CMV promoter was the gift of Dr. H. Ertl (Philadelphia, PA). Plasmid DNA expressing IL-10 under control of the SV40 promoter was provided by Dr. T. Mosmann (Alberta, Canada). Pc DNA3 expressing IFN-gamma was a gift of Dr. S. Huang (Baltimore, MD). Pc DNA 3.1 expressing IL-4 was produced in our laboratory, using IL-4 cDNA purchased from ATCC (Rockville, MD) No. 37561. Plasmid DNA expressing B-galactosidase (pCMV-beta) was purchased from Clontech Laboratories (Palo Alto, CA; No. 6177-1). The expression of different plasmids was checked by enzyme-linked immunosorbent assay (ELISA), enzyme-linked immunospot (Elispot), and Bioassy (IL-4). The vector control for each plasmid was the corresponding vector without cDNA.

Ocular Plasmid DNA Administration : For the generation of immune responses to HSV, 100 ug of gB DNA in 4-5 ul of DNase-free water was dropped on the eyes of 4 week old mice and gently massaged with the eyelids. The DNA immunization was done three times with 10 day intervals. Fifteen days after the last immunization, serum, feces, vaginal washes and tears were collected prior to challenge with HSV, monitoring for death or survival. For the immunomodulation of HSK, 100 ug of plasmid DNA encoding different cytokines in 4-5 ul of DNAse-free water

was applied to the scarified eyes on three occasions (day -3, day +2 and day +5 and with respect to the ocular infection).

Virus Titration : At different times after intravaginal challenge, vaginal washings were collected by pipetting 100 ul of PBS into the vaginal cavity. 50 ul of each individual sample was further diluted and viral titers were obtained by using a plaque assay performed on Vero cells as described elsewhere [10].

Antibody Detection by ELISA : gB-specific IgG, IgG2a and IgG1 were measured in the individual serum collected from different mice by standard enzyme linked immunosorbent assay (ELISA) as described previously [4]. Tears were collected fifteen days after the last immunization by washing the eyes of individual mice with 20 ul of sterile PBS using a pipette. Eye washes from five to ten mice were pooled together. gB-specific IgA was measured in individual fecal samples, vaginal lavage, and pooled tears from different immunized groups by ELISA as described previously [4].

Quantification of Cytokine-producing Cells by ELISPOT Assay : Splenocytes from various DNA or HSV-treated mice were collected fifteen days after the last immunization, and were used as effector cells. Enriched dendritic cells (DC) were obtained from naive female retired breeders as described previously [16]. The responder cells and the DC (naive or pulsed with UV-inactivated HSV) were added at a responder to stimulator ratio of 50:1, 25:1, 12.5:1, and 6.25:1 in 200 ul of RPMI/10% FBS per well into the coated and blocked ELISPOT plates. ELISPOT assay was performed as described previously [4].

Cytokine Quantification by ELISA or Bioassay : Immune splenocytes obtained from various DNA or HSV-recipient mice were screened for their

cytokine profile by an enzyme-linked immunosorbent assay (ELISA) as described previously [4]. IL-4 was detected by bioassay using CT4.S cells (IL-4 and CT4.S cells were gifts provided by Cynthia Watson, National Institutes of Health, Bethesda, MD). Concentrations of the cytokines were derived from the standard curve.

Lymphoproliferation Assay : Immune splenocytes (responder) from various group of mice were collected and restimulated in vitro for four days, with varying ratios of stimulator cells. X-irradiated naive syngenic splenocytes (stimulator) were infected at a multiplicity of infection (moi) of 1.5 with UV-HSV-1 (KOS) and uninfected stimulators were used as the control. Responder-to-stimulator ratios ranged from 10:1 to 0.3 to 1. Lymphoproliferation was measured as described previously [11]. Proliferation index is obtained at a responder to stimulator ratio of 10:1. As a positive control, each responder was stimulated with concanavalin A (5 ug/ml).

Delayed Type Hypersensitivity (DTH) Assay : Two weeks after the last immunization, test antigens in 20 ul of PBS were injected in the ear pinna and the ear thickness was measured with a screw gauge meter (Oditest, H. C. Kroeplin GHBH, Germany) after 48h, as described in detail elsewhere [17]. Test antigens used were UV HSV-1 (KOS) (10^5 $TCID_{50}$ prior to UV inactivation) or Vero extract in the right or left ear, respectively. The mean increase between right and left ear was calculated. The values are represented as n x 10^{-2} mm.

B-galactosidase Staining : Certain times after topical ocular administration of 100 ug of B-gal DNA in 4-5 ul of DNase free water, different organs were collected and fixed with 4% paraformaldehyde in PBS for 1 hr at 4'C. The fixed tissues were washed several times with

PBS and reacted overnight in substrate solution containing 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, 2 mM $MgCl_2$, 120 ul of 10% Nonidet-P-40, and 100 ul of 1% sodium deoxycholate per 20 ml, and 2 mg of 5 bromo-4-chloro-3 indolyl-B-D galactoside (X gal) per ml. After several washings with PBS, whole tissues were fixed with 3.7% formaldehyde in PBS. Tissues were embedded in paraffin, sectioned at 5 um, and stained with nuclear fast red.

Gene Expression Following Topical Ocular DNA Administration : Four days after administration of plasmid DNA encoding B-galactosidase (B-gal) or vector controls, different tissues were removed from each of three animals per experimental group. RNA was isolated from cervical lymph nodes (C.L.N.), mediastinal lymph nodes (M.L.N.) or spleen as described previously [13]. RNA was treated with DNase (20 U/50 ul) for 1 hr at 37'C. After phenol-chloroform extraction and ethanol precipitation, the absence of plasmid DNA was confirmed with PCR. Total cellular RNA was reversed transcribed [13], and B-actin and B-gal were detected by amplification of aliquots of cDNA [4].

Topical Application of Plasmid DNA Encoding gB Induces Systemic and Mucosal Immunity : Anesthetized young mice were exposed to DNA encoding gB or control plasmid by dropping a 4-5 ul volume containing 100 ug of DNA onto the central cornea. Eyelids were closed manually and these were used to gently massage the cornea. Repeat immunizations were performed on days 10 and 20 and samples taken for analysis fifteen days after the third immunization. Control groups were immunized with 10^5 pfu HSV-1 KOS or vector DNA.

RESULTS

The results revealed the development of a serum antibody response in all gB DNA-immunized mice. Furthermore, topically immunized animals also developed both proximal and mucosal immune responses (Table 1). Thus Ag specific IgA was present not only in tears but also in the distal mucosae of the gut and vaginal tracts. The mucosal antibody responses were of the same magnitude as commonly obtained following intranasal immunization with DNA [4]. Interestingly, the serum Ab response reflected a dominant Th1 profile, similarly to that after IM immunization, and was different than reported to occur following DNA administered by gene gun [18]. It was shown previously that IM, DNA immunization induces only marginal mucosal IgA [4] and similar responses were found in tears (unpublished data).

Animals topically immunized with gB DNA also showed antigen-specific T cell responses. These were measured in several different assays. All topically immunized mice developed HSV specific DTH responses (Table 2), a measure of Th1 CD4+ T cells [19]. Specific T cell immunity was also detectable in splenic cells by lymphoproliferation, antigen-induced cytokine release, and by Elispot (Table 2). The latter two assays revealed a response pattern that included both Th1 and Th2 cytokines, but the former appeared as predominate.

Topical Ocular DNA Induced Protective Immunity Measured Systemically and by Mucosal Challenge : To determine if topically immunized mice had functional immunity to HSV, animals were virus challenged both systemically and via the vaginal mucosal surface. To assess systemic immunity, mice were challenged using the zosteriform model employed extensively by our laboratory [19]. This system entails

cutaneous exposure to virulent virus and the observance of subsequent cutaneous lesions. Unprotected mice usually die of viral encephalitis. As recorded in Table 3, the majority of mice immunized topically with gB DNA survived challenge. The level of resistance observed resembled that noted in groups of IM gB DNA-immunized mice [4], and was less than that evident in the virus immunized mice.

Additional groups of mice were also challenged with HSV via the vaginal tract. In such experiments, mice were synchronized into diestrus by progesterone pretreatment, which renders adult animals uniformly susceptible to genital infection with HSV [12]. As shown in Table 4, the majority of animals topically exposed to gB DNA resisted challenge. In addition, gB DNA-immunized animals showed lower titers and a shorter duration of virus secretion than were evident in vector-immunized animals (Table 5). Immunity, however, was less than that observed in animals immunized ocularly with infectious HSV. The ocular route appeared to be as effective as the intranasal route to achieve vaginal immunity to HSV [4].

Topical Ocular Exposure to Cytokine DNA Modulates the Subsequent Herpetic Ocular Disease : Infection of the cornea with HSV leads to the subsequent development of an immunoinflammatory disease called herpetic stromal keratitis (HSK) [20]. This lesion involves the essential participation of Th1 CD4+ T lymphocytes [21]. To test modulation of subsequent ocular CD4+ Th1 response, animals were topically exposed to cytokine DNA prior to HSV infection. For cytokine DNA exposure, the corneas of anesthetized mice were lightly scarified. Groups of mice were topically exposed to either DNA encoding IL-4, IL-10, IL-2 or IFN-gamma and after 3 days were infected ocularly with HSV-1 RE as described previously [13]. Administration of cytokine was repeated at day 2 and day 5 post- infection and infected animals were then evaluated for the

subsequent development and severity of HSK. As is shown in Figure 1, pretreatment with either IL-4 or IL-10 DNA led to a significant diminution in the severity of the HSK response. In many cases, animals failed to develop detectable HSK. However such animals still generated humoral antibody responses to HSV indicating that they had become successfully infected (data not shown). Pretreatment with IL-2 or IFN-gamma DNA had no effect on the development of HSK, although there was an overall trend toward increased disease severity with IL-2 (not significant statistically). Individual blood samples were analyzed at day 20 post-challenge to measure the HSV-specific IgG isotype response in animals exposed to the various cytokines. The results showed that animals in all groups responded approximately the same, but the isotype response profile in IL-4 exposed-mice showed a significantly diminished IgG2a; IgG1 profile (table 6).

Expression Sites For DNA After Topical Application of DNA :
Limited experiments were performed to establish if DNA deposited topically to the cornea reaches to sites beyond the superficial structures of the eye. Protein and mRNA signals detectable using a plasmid encoding B-galactosidase, were evident in draining and distal lymphoid tissues (Fig 2 and 3). Signals were especially strong in the DLN (anterior cervical) but also in the mediastinal LN. Some signals were also evident in the spleen. No evidence of B-galactosidase protein expression was apparent either in ocular tissues or nasal mucosa at least at the 1 and 5 days examination times.

DISCUSSION

We showed that eukaryotic expression plasmids encoding viral Ag delivered topically to the uninjured eye provided an effective way of inducing both systemic and mucosal immunity and represented a means of achieving immunomodulation. Few studies have exploited the ocular surface to induce an immune response because the protective functions of lacrimation and the blink reflex rapidly remove deposited material from the cornea. Pathogens that enter via the cornea often have special adhesion characteristics [22]. Following corneal scarification or direct injection into the conjunctiva, both proteins and peptides may induce systemic and mucosal immune responses, but these tend to be inconsistent [23]. In light of such findings with proteins, it came as a surprise that the topical administration of plasmid DNA routinely led to uptake and the subsequent generation of an immune response to plasmid encoded antigen. How such topically administered DNA gains access to the antigen presentation machinery of the immune system remains to be shown. Access of the DNA through the corneal epithelium, which is the non-irritated cornea, lacks APC such as Langerhans cells, is unlikely [23]. Nevertheless, we favored the notion that the chief route of access may be through the conjunctival epithelium, a structure rich in APC such as dendritic cells and macrophages [25]. The fact that the induced immune response was mucosal, including distal mucosal, as well as systemic, supports the conjunctiva access hypothesis. Thus the conjunctiva is recognized to form part of the common mucosal immune system [26].

An alternative route of DNA entry may be through the nasal mucosa, since the conjunctival surface drains to the nasal cavity via the canal of Schlem [26]. In support of this idea, plasmid expression signals were present in the mediastinal lymph node. The distribution of plasmid

signals (B-gal) were, in fact, unexpectedly widespread following topical ocular administration and even included the spleen. Further studies are underway to identify trafficking routes and their kinetics in order to understand how DNA administered to the body surface can successfully achieve immune induction and modulation.

Although it might be impactical to advocate using the eye as an approach for immunization, but the fact that topical application of DNA encoding cytokines led to expression and functional effects suggests that the cornea could provide a valuable site to achieve immunomodulation. So far we have only tested this notion using a model disease process (HSK) an immunoinflammatory lesion in the corneal stroma that occurs in susceptible mice as a sequela of HSV infection [27]. A similar disease occurs in man, although in this case lesions usually follow HSV reactivation [28]. The human disease is one of the most important infectious causes of vision impairment in the developed world [28]. The murine disease is recognized to be an immunopathological process orchestrated by CD4+ Th1 T lymphocytes [27], and is therefor, an acceptable model for studying the pathogenesis of HSK.

In our immunomodulation studies, we showed that preexposure of the eye to two tested cytokine DNAs - IL-10 and IL-4 diminished or even prevented the subsequent development of HSV induced lesions. We interpret these observations to mean that the disease modulating cytokines either abrogated the induction of the CD4+ Th1 response or diminished the recruited inflammatory response. It is also likely that both mechanisms occur and that the immunomodulatory cytokines function in different ways.

Preliminary evidence supported the fact that IL-4 pretreatment served to shift the response towards the Th2 pattern, an effect not observed after IL-10 pretreatment. In the case of IL-10, the disease suppression may have been associated with inhibition of inflammatory cell recruitment or their secretion of tissue injuring mediators. Thus in our previous studies using a therapeutic model, only IL-10, and then only when administered topically to eye, achieved lesion remission [5]. IL-4, effective as an immunomodulator when given prophylactically, was without effect therapeutically. Clearly more studies are required to define the mechanism of immunomodulation by ocularly administered cytokines and to determine if the ocular route can be exploited to achieve modulation at distal organs.

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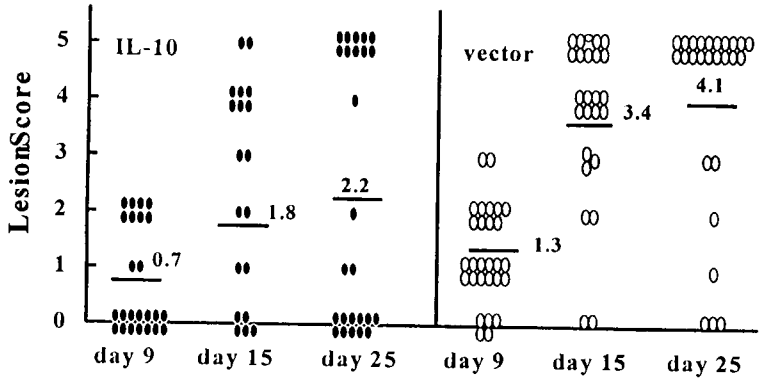
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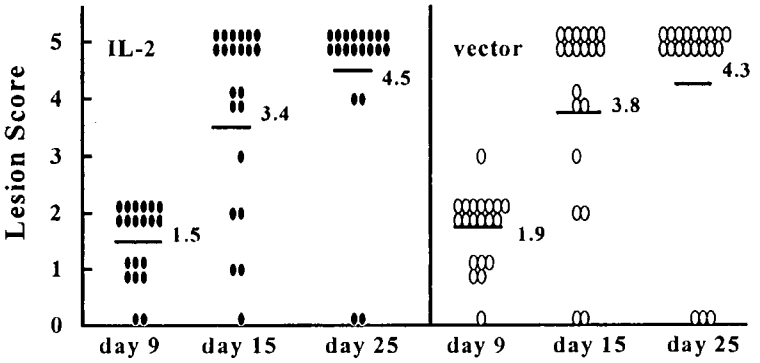
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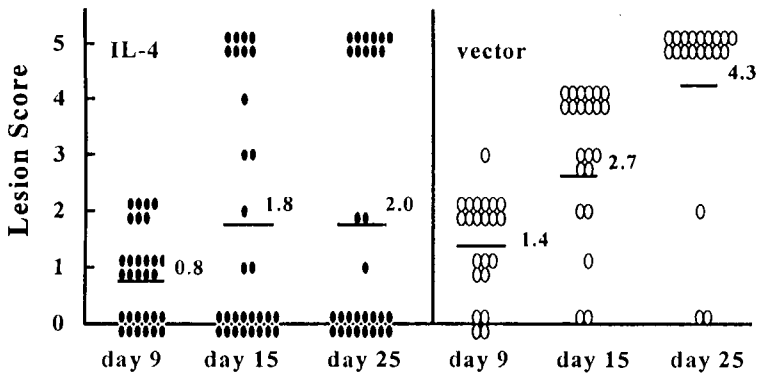
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Figure 1: **Effect of topical ocular administration of cytokine DNA on herpetic ocular disease**. Groups of animals were given 100 ug each of IL-10, IL-2, IL-4, or IFN-gamma or respective vector controls ocularly at d-3, d2 and d5 with respect to the ocular infection. At day 0, 10^6 PFU of HSV-1 RE were placed on the cornea and massaged with the eye lids. Animals were examined daily after infection by slit lamp biomicroscopy, and the severity of the stromal keratitis in each cornea was graded form 0 to 5.


Lesion Score
IL-10
vector
0.7
1.8
2.2
1.3
3.4
4.1
day 9
day 15
day 25
day 9
day 15
day 25


Lesion Score
IL-2
vector
1.5
3.4
4.5
1.9
3.8
4.3
day 9
day 15
day 25
day 9
day 15
day 25


Lesion Score
IL-4
vector
0.8
1.8
2.0
1.4
2.7
4.3
day 9
day 15
day 25
day 9
day 15
day 25

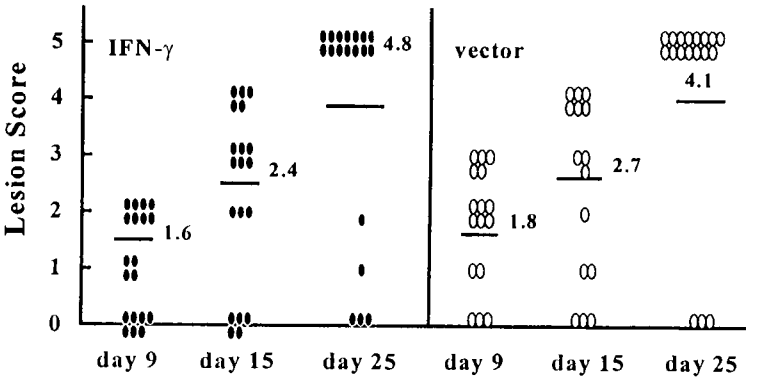

Lesion Score
IFN-γ
vector
1.6
2.4
4.8
1.8
2.7
4.1
day 9
day 15
day 25
day 9
day 15
day 25

Figure 2: **Detection of B-gal protein in different tissues**. Five days after topical ocular administration of 100 ug of plasmid DNA encoding B-gal or vector DNA, different tissues were removed, fixed with 4% paraformaldehyde, and incubated overnight with X-gal substrate at 37'C. Tissues were fixed in 37% formaldehyde, embedded in paraffin, sectioned at 5 um, and stained with nuclear fast red. (A) Sections A, C and E are spleen (X340) mediastinal LN (X340) and LN (X340) of mice which received B-gal DNA topically. Sections B, D and E are the same organs from which received vector DNA.

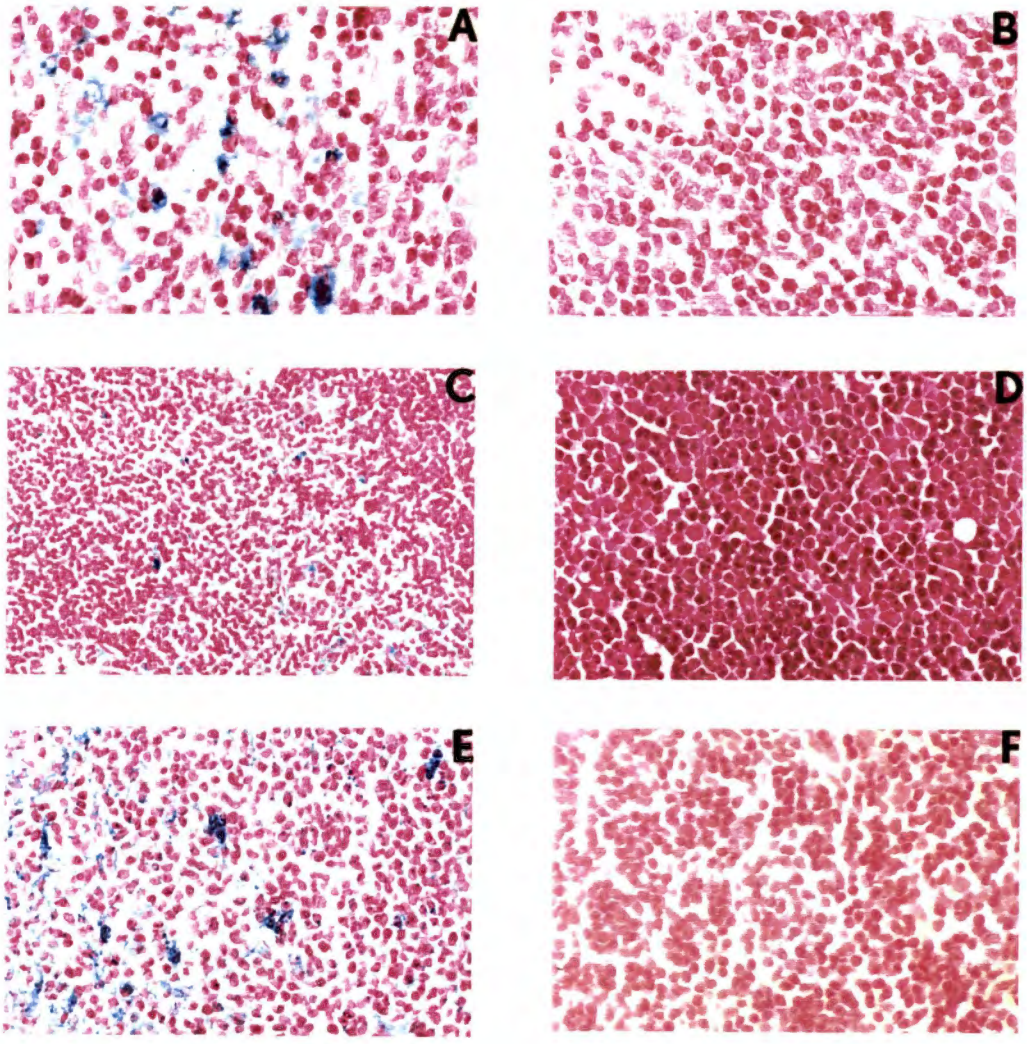

A
B
C
D
E
F

Figure 3: **Qualitative profile of B-gal and B-actin (housekeeping gene) expression in C.L.N., M.L.N., and spleen**. Different tissues were removed 4 days after topical ocular administration of 100 ug of plasmid DNA encoding B-gal or vector DNA (3 mice per group). RNA was isolated and reverse transcribed and the equal amount of cDNA was used for detection of B-gal and B-actin using the specific primers. Lanes: M, 1.0 kb DNA ladder; 2 and 3, C.L.N.; 4, 5; M.L.N.; 6 and 7 spleen. Lanes 2, 4 and 6; samples from B-gal DNA immunized mice and 3, 5, and 7; samples from vector administered mice.

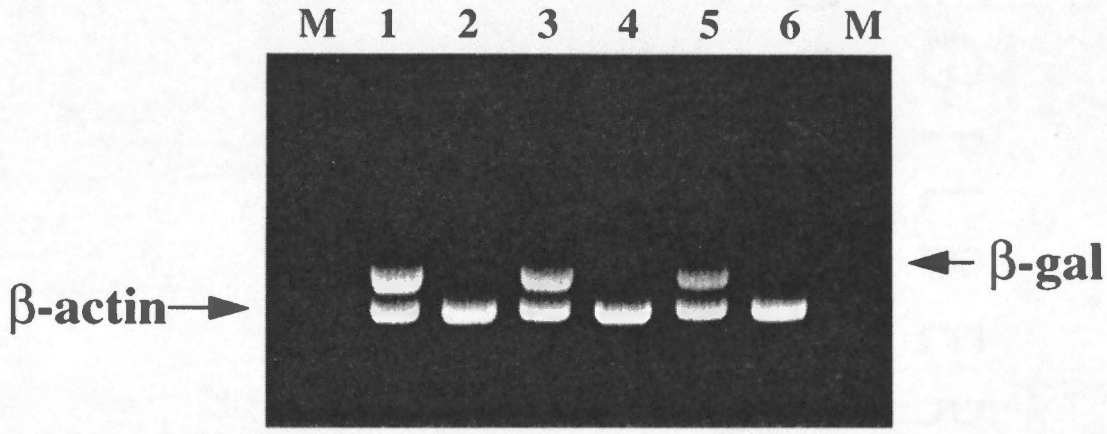

M 1 2 3 4 5 6 M
β-actin
β-gal

Table 1. **Systemic and mucosal gB specific antibody responses following topical gB DNA immunization or ocular virus infection**

Topical immunization [a]	Serum IgG (ng/ml)	IgG_{2a}/IgG_1 ratio	Ocular IgA (ng/ml)	Fecal IgA (ng/ml)	Vaginal IgA (ng/ml)
gB DNA	70 ± 41 (8/8)	3/1	32 ± 9	21 ± 6 (7/8)	16 ± 5 (6/8)
vector DNA	<5	0	0	0	0
HSV-1	5380 ± 332 (5/5)	12/1	690 ± 130	165 ± 61 (5/5)	831 ± 210 (5/5)

[a]100ug of DNA Plasmid or 10^6 live HSV were applied topically to the cornea. Immunizations were done 3 times at 10 days interval and 15 days after the last immunization, different samples were collected. The levels of gB specific antibodies were measured either in individual mice (serum, feces, and vaginal lavage)or from pooled samples (ocular lavage) by ELISA as described in materials and methods. SD is based on five to eight different samples or three independent experiments (pooled ocular IgA).

[b]Data in parenthesis are number of positive samples/tested samples.

Table 2. **Cellular immune responses in mice topically immunized with gB DNA, vector DNA or with HSV-1**

Immunogen[a]	DTH[b]	Stimulation[c] index	ELISPOT[d] (SPC/10^6 cells)			ELISA[e] and Bioassay (pg/ml)		
			IFNgamma	IL-4	IL-5	IFNgamma	IL-4	IL-5
gB DNA	6.6 ± 1.8	10.5a	164 ± 29	15 ± 7	10 ± 3	675	<50	31
vector DNA	0	1.3b	21 ± 4	4 ± 2	1	83	<5	0
HSV-1	11.0	25.4c	298 ± 63	28 ± 7	11 ± 5	1550	50	12

[a]BALB/c were topically immunized with gB DNA or HSV-1 (KOS) at days 0, 10 and 20. Fifteen days after the last immunization different parameters were analyzed.

[b]Delayed-type hypersensitivity (DTH). Data present the mean difference (10^{-2} mmV) and the left ear pinnae in each group at 48h post challenge was described in Materials and Methods (a vs. b, $p < 0.001$, a vs. c $p = 0.08$ and b vs. c, $p < 0.001$).

[c]HSV-specific lymphoproliferation assay: proliferation index was calculated based on the ratio of counts per minute of responders plus HSV-infected stimulators and responders plus naive stimulators as described in Materials and Methods. Data is one of the four representative experiments.

[d]ELISPOT assay: The number of spot-producing cells (SPC) is determined by ELISPOT as mentioned in Materials and Methods. Values represnt average from three independent experiments.

[e]ELISA and Bioassay: culture supernatants collected at 72h were screened for cytokines IFN-gamma and IL-5 by ELISA and for IL-4 by CT4.S Bioassay. The data represent one of the three representative experiments.

Table 3. **Protection from cutaneous challenge in ocular DNA or virus immunized mice**[a]

	gB DNA		vector DNA		HSV	
	EXPT 1	EXPT 2	EXPT 1	EXPT 2	EXPT 1	EXPT2
no. of mice survived/no. of mice challenged	6/8	5/8	1/6	0/5	4/4	4/4
% of protection[b]	68		9		100	
average time of death ± SD (days)	9.6 ± 1.7		8.3 ± 1.5		á[c]	

[a]Fifteen days after the last ocular immunization with gB DNA, vector control or 10^7 PFU of live HSV-1(KOS) BALB/c mice were challenged intracutaneously with 2 10^5 PFU of HSV-1.17. Animals were inspected daily for the development of zosteriform ipsilateral lesions, encephalitis and death. Animals in distress were sacrificed.

[b]Average of both experiments.

[c]No animal died until the end of the experiments.

Table 4. **Protection from vaginal challenge in ocular DNA or virus immunized mice**[a]

	gB DNA		vector DNA		HSV	
	EXPT 1	EXPT 2	EXPT 1	EXPT 2	EXPT 1	EXPT 2
no. of mice survived / no. of mice challenged	4/6	6/10	0/5	0/5	5/5	4/4
% of protection[b]	64		0		100	
average time (days) (days)	8.3 ± 1.3		5.8 ± 1.5		á[c]	

[a]BALB/c mice were immunized oculary with 100ug of gB DNA, vector control or 10^7 PFU of live HSV-1 (KOS). Fifteen days after the last immunization, the mice were injected with 2 mg of DP per mouse. The mice were challenged, five days after the administration of DP, intravaginally with $2x10^5$ PFU of HSV-1 Mckrae. The mice were observed daily for vaginal inflammation and death. Animals in distress were sacrificed.

[b]Average of both experiments.

[c]No animal died until the end of the experiments.

Table 5. **Viral titer after vaginal challenge in ocular gB DNA or virus immunized mice**[a]

Immunization	Log_{10} of vaginal viral titer at day postchallege		
	1	3	5
gB DNA [a]	5.1 ± 0.2 (5/5)[b]	4.0 ± 0.1 (5/5)	3.3 ± 0.2
vector DNA [b]	5.9 ± 0.4 (5/5)	5.1 ± 0.2 (5/5)	4.4 ± 0.3
HSV-1 (KOS) [c]	2.2 ± 0.2 (4/4)	0 (0/4)	0 (0/4)

[a]Fifteen days after the last immunization, mice were infected with $2x10^5$ PFU of HSV-1 McKrae. Vaginal lavages were collected at days 1, 3 and 5 to determine vaginal viral titers. At day 1, c vs. a or b $p< 0.001$ and at d 3 and 5 a vs. b $p < 0.001$.

[b]Data in parenthesis are number of vaginal washings positive for virus/total number of vaginal samples tested.

Table 6. **Effect of topical cytokine DNA exposure on antibody response to ocular HSV infection**[a]

Plasmid DNA encoding cytokine	Total IgG (mg/ml)	IgG2a (ng/ml)	IgG1 (ng/ml)	IgG2a /IgG1 (ng/ml)
vector	47.7 ± 8.8	33280 ± 10565	905 ± 145.	36.8
IL4	48.5 ± 12.7	16086 ± 2558	1368 ± 768	11.7
IL10	36.5 ± 14.2	22995 ± 9706	915 ± 422	25.1
IL2	67.3 ± 16.5	41402 ± 9268	877 ± 172	47.2
IFN-g	58.7 ± 16.4	39860 ± 6395	1090 ± 176	36.6

[a]Mice were topically (ocular) given 100 ug of expression DNA encoding different cytokines in 4-5 ml of DNase free water. The administration was done at d-3, d2 and d5 with respect to the ocular infection with 10^6 PFU of HSV-1 (RE). Twenty days after virus infection, individual serum samples were collected from different groups of mice, and HSV-specific antibodies (total IgG, IgG_{2a}, and IgG_1) were measured as described in Materials and Methods.

PART V

CONCLUSIONS

In the first part of this dissertation, the pathology of HSK was reviewed. Some of the early events in the pathogenesis of HSK were further characterized, including the role of specific inflammatory molecules. Early induction of TNF-alpha, IL-12 and iNOs in the stroma of HSV-1 infected animals was reported. These findings help to elucidate a better understanding of the pathogenesis of HSK in the murine model. The early production of TNF-alpha seems likely to act as a chemo-attractant, IL-12 acts as an inducer of Th1 development in the cornea and iNOs has multiple functions including viral clearance and / or tissue destruction.

In the second section, various experiments were designed to protect the cornea from destruction, following HSV-1 infection. A new technique of local protein expression by somatic delivery of a plasmid vector into the ocular tissues was developed. It was found that overexpression of cytokine IL-10 protected up to 75% of infected eyes from necrosis. Sustained expression of IL-10 overcame an established ocular infection during the early stages of disease (high neovascularization, cell infiltration and edema).

In the third section, the experiments were geared toward further characterization of these phenomena, to understand if the ocular gene delivery of HSV-1 specific viral glycoprotein (gB) could induce a protective immune response. These data revealed generation of mucosal and systemic immunity as measured by different approaches. This immunity was strong enough to protect the majority of animals following lethal mucosal or systemic challenge. Following topical ocular administration of DNA, the protein expression was evident at distal sites (CLN, MLN, and the spleen).

From the above experiments, it can be concluded that direct gene delivery can be used as a novel tool in the management of ocular immunopathology caused by HSV-1. In conclusion, it is hoped this new approach might be developed and applied to other inflammatory disease processes where convenient immunomodulation is not currently available.

VITAE

Massoud Daheshia received a Bachelor of Science degree in Biochemistry in 1986 from the University of Jussieu (PVII) in Paris.

He continued his studies at the same university and obtained a masters degree in Biochemistry in 1988. To become better trained in molecular biology he entered the Molecular Pharmacology program and obtained a D.E.A. in 1989, from the University of Pierre and Marie Curie (PVI) in Paris. He then worked until 1992 as an RA on various molecular mechanisms of gene regulation by hormones.

In 1994 Mr. Daheshia entered the Department of Comparative and Experimental Medicine at the University of Tennessee, Knoxville. He soon started work in Dr. Rouse's Immunology Laboratory, investigating the pathogenesis of HSK. He obtained his Ph.D. degree in December 1997.