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HERPES SIMPLEX VIRUS INDUCED STROMAL KERATITIS: ROLE OF T-LYMPHOCYTE SUBSETS IN THE IMMUNOPATHOLOGY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Charles K. Newell

May 1989

This thesis is dedicated to my parents. Without their strong moral and financial support I could have never achieved the level of education I have so deeply desired.

ACKNOWLEDGMENTS

Many thanks to the members of my advisory committee, Drs. Carl J. Wust, and Robert N. Moore.

I feel especially privileged to have been acquainted both professionally and personally with Dr. Deborah Sendele. Thank you for everything Deb.

A special thank you to Drs. Stephen G. Norley, and Steve Martin, who provided an extraordinary amount of assistance and supervision throughout my graduate education. These two outstanding gentlemen provided not only myself, but the entire laboratory with a special enthusiasm for the study of Cellular Immunology. They also provided many interesting evenings of discussion at Vic and Bills.

I wish also to thank my major advisor, Dr. Barry T. Rouse for allowing me the opportunity to work under his knowledgeable guidance. Dr. Rouse has made a significant contribution to the success I hope to achieve in life.

ABSTRACT

Herpetic stromal keratitis (SK), a frequent cause of visual impairment, is considered to represent an immune-mediated inflammatory response to persistent herpes simplex virus virions or subcomponents within the corneal stroma. The experimental disease in mice involves the essential participation of T lymphocytes, but the role of T lymphocyte subsets in either mediating or controlling the disease is uncertain. In this report,rat monoclonal antibodies were used to selectively deplete mice *in vivo* of $CD4^+$ (helper-inducer) and $CD8^+$ (cytotoxic-suppressor) T cell populations and the effect on herpetic SK was evaluated. As measured by flow cytometry, mice treated with anti-CD4 monoclonal antibody (GK1.5) were >95% depleted of $CD4^+$ T lymphocytes and mice treated with anti-CD8 monoclonal antibody (2.43) were 90% depleted of $CD8^+$ T lymphocytes. Depleted and nonspecific mouse ascites treated control mice were infected topically on the corneas with herpes simplex virus type 1, and the induction of various immune parameters during the acute infection was evaluated. $CD4^+$ depleted mice failed to produce either a significant antiviral antibody or delayed-type hypersensitivity response but were capable of producing normal cytotoxic T lymphocyte responses. In contrast, $CD8^+$ depleted mice produced antiviral antibody and delayed-type hypersensitivity responses comparable with those in control animals, but cytotoxic T lymphocyte responses were markedly reduced. Clinical observations of the corneas revealed that SK in $CD4^+$ depleted mice was significantly reduced, whereas in $CD8^+$ depleted mice SK developed more rapidly, was more severe, and involved a greater percentage of mice. These observations implicate the $CD4^+$ T lymphocyte subset as the principal mediators of SK and $CD8^+$ T lymphocytes as possible regulators that control the severity of SK.

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ABBREVIATIONS

HSV-1 = Herpes Simplex Virus type-1

SK = Herpetic Stromal Keratitis

TC = Topical Corneal

AC = Anterior Chamber

PMN = Polymorphonuclear Leukocyte

DTH = Delayed Type Hypersensitivity Response

CTL = Cytotoxic T-Lymphocyte

FACS = Fluorescense Activated Cell Sorter

CNS = Central Nervous System

$TCID_{50}$ = Tissue Culture Infectivity Dose - 50% end point

Chapter I

LITERATURE REVIEW

Introduction

Infection of the eye with Herpes Simplex Virus (HSV) can cause a variety of syndromes, but perhaps the most significant, because it frequently causes permanent vision impairment, is Herpetic Stromal Keratitis (SK). In fact, this disease is amongst the most common causes of unilateral corneal opacification in the United States and other developed nations (1). Approximately 500,000 new cases of HSV ocular disease occur annually (2), and longitudinal studies have suggested that up to 25% of patients with the epithelial form of herpetic keratitis will eventually develop SK (3). A significant proportion of the individuals who develop SK will then develop a reoccurrence of the disease within 2 years of the primary attack (4,5) and it is the reccurrent nature of this disease which contributes to corneal scarring, opacification and the eventual loss of vision. The damaging effects of SK is not attributable to replicating HSV within the corneal epithelium or stroma. Instead much evidence indicates that the damage is primarily caused by the host's immune response to persistent non-replicating viral particles within these tissues (6-15). Support for this hypothesis comes from both clinical and experimental evidence.

Clinical Evidence for Immunopathology in SK

Clinical evidence that SK represents an immunopathological disease comes from the finding that antiviral therapy is ineffective in the treatment of this disease (15), whereas much success in limiting the disease is achieved using

immunosuppressive drugs (16). Furthermore, it is not possible to isolate infectious virus particles from the cornea once the disease has manifested itself. Utilizing immunofluorescent microscopy Metcalf and Kaufman (10) showed the presence of viral antigens in keratocytes of the cornea during an attack of SK, however, electron microscopy and viral culture assays of these isolated tissues failed to show mature infectious viral particles. This same study determined that the predominate infiltrating cells in these tissues were T-lymphocytes and many of these cells were in intimate contact with infected keratocytes that were undergoing varying degrees of degeneration. This was the initial report which suggested a T-lymphocyte attack on corneal cells infected with HSV (10).

Experimental Evidence for Immunopathology in SK

The first indictment of T-lymphocytes being principally involved in the pathogenesis of SK was made by Metcalf et. al. (9). In these experiments, athymic mice where challenged by the topical corneal (TC) route with HSV-1 and the subsequent nature of the disease in these animals was compared to their euthymic littermates. The congenitally athymic nude mouse is deficient in T-lymphocytes and thymus dependent immunological functions (17), therefore, a study of SK in athymic mice would allow these researchers to evaluate the importance of T cell immune functions in the immunopathogenesis of SK. The mean severity of SK in athymic mice was similar to that seen in euthymic mice until approximately one week post-infection. Following this time many athymic mice showed signs of neurological disease and eventually succumbed to the virus infection. However, stromal opacity in the surviving athymic mice did not progress, but instead the disease subsided to eventual total clearing of corneal opacity. A minor proportion of the euthymic mice died following corneal challenge with HSV-1, however, the levels of SK in these animals progressed

to severe SK immunopathology. Histological examination of representative euthymic mice eyes revealed the presence of numerous inflammatory cells, polymorphonuclear leukocytes (PMN) and mononuclear cells. In support of previous findings, the mononuclear cells were often found in intimate contact with stromal keratocytes. On the contrary, relatively few inflammatory cells were found in virus infected corneas of athymic mice. The result of this study provided the first experimental evidence that T-lymphocytes play an important role in the pathogenesis of SK, however, many questions regarding the mechanistic nature of this disease remained unanswered. For example, the finding that T-lymphocyte depleted animals produce antibodies against some antigens but not others suggests that an interaction between T and B-lymphocytes is required for antibody synthesis in some instances (18). Therefore, this study was unable to rule out antibodies as participants in the induction of SK, as opposed to T-lymphocyte mediated cytolysis alone. Furthermore, an essential role for sensitized lymphocytes in the production of chemotactic lymphokines for PMN has been established (19), and this study could not rule out a requirement for PMN in SK immunopathology.

Role for PMN in SK Immunopathology

The role of PMN and immune complexes in the development of SK immunopathology was addressed by Meyers-Elliot et. al. (11). In this study rabbits were treated with anti-PMN antisera or purified cobra venom factor (CVF) prior to TC challenge with HSV-1. Treating animals with anti-PMN resulted in total depletion of circulating PMN and following corneal infection with HSV-1 there was complete abrogation of structural injury to the corneal stroma. Treatment with purified CVF beginning 3 days after HSV-1 infection depleted animals of the C3 complement component and the resulting corneal stroma inflammation was greatly reduced, as compared to untreated controls.

The result of this study supported the immunopathological nature of SK, however it also revealed that this immunopathological disease was complex and likely involved both cellular and humoral aspects of immunity.

Role for T-lymphocytes in SK Immunopathology

Further support for the role of T-lymphocytes in SK immunopathology, was provided by the adoptive transfer studies of Russell and coworkers (20). This study compared the resultant corneal pathology of normal mice to athymic mice which were reconstituted with normal or HSV-1 immune T-lymphocytes. As discussed previously, athymic mice are genetically unable to mount T-lymphocyte responses and they are also resistant to SK immunopathology. When these animals were reconstituted with normal or HSV-1 immune syngeneic splenocytes these animals were made susceptible to the disease and underwent stromal scarring reactions similar to their euthymic littermates. More dramatic results were observed when the donor cells were obtained from HSV-1 immune animals, cultured in vitro for 5 days, and restimulated with HSV-1 prior to transfer into recipient animals. In fact, the immunopathology was judged to occur more rapidly, and was more severe in these animals as compared to their euthymic littermates. Treatment of the donor cells with anti-T-lymphocyte antisera plus complement prior to transfer completely abrogated the subsequent immunopathological response. This population of HSV-1 immune splenocytes were extensively characterized and determined to consist predominantly of T-lymphocytes which express high levels of HSV-1 specific H-2 restricted cytotoxicity, contain T-helper cell activity, and were also capable of mediating the delayed type hypersensitivity (DTH) reaction. Thus, Russell and coworkers confirmed the findings of Metcalf et. al. and provided further convincing evidence that T-lymphocytes play an important role in

SK immunopathology. Nevertheless, there remained many questions as to the actual mechanism of the disease. For example, tissue damage may have been mediated directly by cytotoxic T-lymphocytes, or by HSV-1 specific T cells mediating an inflammatory DTH reaction. Furthermore, these T cells may have acted as helper cells in reconstituting athymic mice to produce antibody, which as mentioned previously (11), had been implicated as having a role in the immunopathology of SK.

Role for DTH in SK Immunopathology

As a means of studying the underlying mechanism of SK immunopathology, many investigators have taken advantage of the unique immune responsiveness of mice to HSV following various routes of inoculation. Thus, following a subcutaneous challenge with HSV mice are able to mount the normal repertoire of cellular and humoral immune responses to the virus or viral antigens, e.g., cytotoxic T cells (CTL) are induced, a vigorous DTH response occurs, and normal antibody responses are mounted. However, following inoculation of HSV via the intraperitoneal (22), intravenous (23), or intracameral route (24), or by exposure of mice to certain heterologous anti-idiotypic antisera (25), a unique immune response, termed split-tolerance, occurs whereby a DTH response does not occur but all other measurable aspects of immunity remain intact. Using these various immunization strategies investigators have implicated a role for T lymphocytes acting via the DTH response as the principal means by which the SK immunopathology occurs (8,21). In the case of HSV, this response is mediated by the T-helper cell (26, 27, 28), which is distinguished from its T-lymphocyte counterparts by the expression of CD4 cell surface antigens. Williams et. al. (21) noticed that rabbits which were previously subcutaneously inoculated with live HSV-1 and then subsequently challenged on the cornea with HSV-1 underwent significantly increased

incidences of SK as compared with animals that had not been presensitized. Furthermore, the onset of the disease was more rapid in these animals as compared to controls. In support of this finding was the study by Lausch et. al. (8) whereby mice where tolerized for the DTH response by inoculating HSV-1 intravenously 7 days prior to corneal challenge. These pre-tolerized animals did not develop DTH responses and the nonresponsiveness was shown to be long lasting (at least 6 weeks) and specific for HSV-1. One week following corneal infection none of the DTH-unresponsive mice exhibited clinical or histological signs of ocular pathology. In contrast, mice not intravenously pretreated with HSV-1, showed obvious signs of ocular pathology 7 days post-infection. Additional experiments using different strains and various doses of HSV-1 for the corneal challenge revealed that DTH unresponsive mice remained resistant to SK immunopathology. The results of these two investigations determined that DTH responsive animals could undergo SK immunopathology, but when animals were unable to mount a DTH response then they were spared the immunopathogy normally associated with ocular HSV infections. For the first time, these experiments implicated a mechanism by which SK immunopathology occured, and that mechanism appeared to be at least related to a DTH response within the corneal stroma, and this reaction was likely mediated by CD4+ T-lymphocytes.

Role for CTL in SK Immunopathology

Recently a role for CTL as mediators of SK immunopathology has been proposed by Hendricks et. al. (29, 30, 31). In their initial report these investigators demonstrated that the A/J strain of mice, a strain that is normally highly susceptible to HSV-1 corneal lesions (32) could be rendered resistant to the induction of T-lymphocyte mediated immune responses to HSV-1 antigens (29). The tolerization to HSV-1 antigens was performed by injecting

live HSV-1 into the anterior chamber (intracameral inoculations) of mice eyes 20 minutes after a TC inoculation of the same animal. These dual inoculations of animals resulted in abrogation of the DTH response, and purportedly also suppressed the CTL response. Furthermore the animals were protected from the corneal stromal lesions that normally result from a TC infection alone. Once again the Hendricks study supported a role for T-lymphocytes in SK immunopathology, but which one of the two known subsets of T-lymphocytes (CD4+, CD8+) had specific roles in the disease remained undetermined. In a follow up study, Hendricks and Glorioso (30), reported that simultaneous TC infection with wild-type HSV-1 and anterior chamber (AC) injection of a mutant HSV-1 that does not express glycoprotein C (gC-) results in a unique pattern of immune reactivity to HSV-1 which is characterized by CTL unresponsiveness but a normal DTH response. According to these investigators this treatment conferred significant protection against HSV-1 stromal disease. The AC injection of HSV-1 mutants which lack glycoprotein B (gB-) also failed to induce DTH tolerance, but unlike mice which recieved gC- mutants they only partially suppressed the CTL response, and did not confer protection against HSV-1 stromal disease. Recent studies by the same investigators (31) followed the clinical, histological, and immunohistochemical development of corneal stromal lesions in A/J mice that were either infected topically with wild type HSV-1 or gC- mutant HSV-1 and compared this to animals given simultaneous TC and AC injections of wild type HSV-1. Within one week post-infection, all three groups of mice developed an early inflammatory response in the corneal stroma which consisted of a predominantly granulocytic infiltrate and corneal swelling. In the TC plus AC infected group and in the group receiving only TC infection with the gC- HSV-1, the inflammation quickly subsided and the corneas appeared normal within 3 weeks. The corneal pathology in these mice compared to the pathology observed in animals that received only TC infection with wild-type HSV-1 was

very different. In the latter group, the corneal inflammation observed by one week progressed to corneal neovascularization. In addition a predominant mononuclear cell infiltrate was observed, and massive destruction of the corneal stroma occured within 3 weeks of infection. The results of these studies led the investigators to conclude that an immunopathogenic role for DTH could now be ruled out and they suggested instead that HSV-1 specific CTL were the putative T-lymphocyte subset responsible for HSV-1 SK in the murine model.

Rationale for these studies

The numerous studies of HSV-1 induced SK, reviewed above, have provided substantial evidence to implicate T-lymphocytes as the essential participants in this immunopathological disease. There is less certainty, however as to which T-lymphocyte subset is principally involved in mediating the disease. From these studies it appears that one explanation as to the nature of the disease may not suffice, and consequently there may be multiple mechanisms of immunopathology occurring in HSV-1 induced SK. To readdress the question of the role of one or the other of the two major phenotypic subsets of T-lymphocytes in the immunopathology of SK, this report took advantage of the recently available immunosurgical approach, in which mice can be selectively depleted of a given T-lymphocyte subset by the in vivo administration of monoclonal antibody (MAb) to their appropriate cell surface antigens (27, 33). This well characterized immunosurgical approach has been used to study the role of T-lymphocyte subsets in a variety of different disease and animal models (34-42) and has provided investigators with valuable insight into the nature of many immunopathological diseases. Although the primary goal was to study a variety of immunological responses in T-cell subset depleted mice, the first reported use of immunosurgery in the study of an HSV immune

response was made by Cobbold and coworkers (32). These investigators presented convincing evidence that selective depletion of the major murine T-lymphocyte subsets could be achieved by the *in vivo* administration of rat IgG2b MAb. They determined that therapy using anti-CD8 and anti-CD4 MAb would cause at least a 96% reduction of these T-lymphocyte subsets in the spleen and lymph nodes, and this substantial reduction in T-lymphocytes was also long lasting (up to 5 weeks in non-thymectomized animals). Within 10 days of the last MAb treatment, it was not possible to detect rat immunoglobulin in these mice, however there was a detectable anti-globulin response. These results indicated that the suppression was unlikely to be due to blocking of function, or temporary changes in the pattern of lymphocyte circulation. The indications were that the loss of T-lymphocyte subset function was the result of selective depletion of the particular T-cells. In this report mice depleted of CD4+ cells were unable to make antibodies to sheep red blood cells, and were also unable to develop a DTH reaction to HSV-1. From this information emerged a new and exciting means by which one could study the role of one or the other T-lymphocyte subset in the immune response to HSV. The relative ease and inexpensiveness, are additional reasons this method is preferred over the adoptive transfer method to study the role of T-cell subsets in a variety of immune responses.

Theiler's murine encephalomyelitis virus (TMEV) gives rise to a biphasic disease which in the early phase (within one month), resembles poliomyelitis, and in the late phase causes an inflammatory demyelination of the central nervous system similar to that seen in victims of multiple sclerosis. In an attempt to study the role of CD4+ T-lymphocytes in the acute and chronic phases of TMEV infections, Welsh et. al. (33) employed the immunosurgical technique to deplete these cells from mice prior to infection and prior to the onset of clinical signs of demyelination. Mice treated with anti-CD4 prior to the onset of

disease failed to produce antibodies to TMEV and were unable to clear the virus from the CNS. These animals subsequently died within the first month of infection. However, if mice were depleted of CD4+ T-cells before the demyelination phase of disease, a decrease in the incidence of disease was observed in 77% of the normal mice to 36% of CD4 depleted mice. These results, and the finding that immunocompetent animals developed antibodies against myelin suggested that autoimmune mechanisms, possibly mediated by CD4+ T-lymphocytes, played a role in TMEV-induced demyelination.

Recently, the immunosurgical approach was used to study the role of CD4+ T-lymphocytes in the generation of toxoplasmic encephalitis in mice chronically infected with Toxoplasma gondii (34). The results of this study revealed that the *in vivo* administration of anti-CD4 to these infected mice significantly reduced the inflammatory response in the brains of the animals. Furthermore, a recrudescense of the inflammatory process occurred following the discontinuation of treatment with anti-CD4. The experiments were performed with different strains of mice and T. gondii, and similar results were obtained. Similar to the study above, these investigators concluded that the CD4+ T-cells in this disease model were the likely mediators of the inflammatory process which caused immunopathology in the brains of these chronically infected mice.

Utilizing the immunosurgical approach to selectively deplete mice of a particular T-lymphocyte subset, this report demonstrates the ability to deplete greater than 95% of CD4+ and 90% of CD8+ T-cells by the in vivo administration of MAb from the hybridomas; GK1.5 (anti-CD4) and 2.43 (anti-CD8). The effect of this therapy on the clinical outcome of HSV-1 induced SK, as well as a variety of other immune functions were addressed. Thus, depletion of CD4+ T-lymphocytes resulted in a profound reduction of SK immunopathology whereas the depletion of CD8+ T-lymphocytes resulted in SK immunopathology which developed

more rapidly, was more severe, and involved a greater percentage of mice. Furthermore, SK could be reconstituted in CD4 deficient mice following the adoptive transfer of HSV-1 immune T-lymphocytes. The treatment of SK immunopathology using anti-CD4 was also possible if treatment was initiated prior to or during the onset of the disease, whereas it was not possible if treatment was initiated following the development of disease. Therefore, this report provides evidence which implicates the CD4+ T-lymphocyte subset (helper/inducers) as principally involved in the SK immunopathology, and also demonstrates an essential role for CD8+ T-lymphocytes (cytotoxic/suppressor) in controlling the severity of SK This report also hypothesizes that the mechanism by which SK immunopathology occurs is by a CD4+ T-cell mediated DTH reaction within the corneal stroma which is controlled by CD8+ T-cells.

Chapter II

INTRODUCTION

Infection of the eye with herpes simplex virus (HSV) can cause a variety of syndromes, but perhaps the most significant, because it frequently causes permanent vision impairment, is stromal keratitis (SK), which is associated with recurrent episodes of HSV infection (39). Although the exact nature of the pathogenesis of SK remains unresolved, much evidence supports the involvement of an immune mediated inflammatory process (6-14). Thus, whereas antiviral agents are usually ineffective in the treatment of SK (15), this disease often responds well to immunosuppressive therapy (16). The notion that SK represents an immune mediated inflammatory response to persisting nonreplicating viral components or corneal neoantigens receives much support, but there is less certainty about the mechanistic nature of the immunopathological reaction (39). Studies on experimental animal models of SK support a role for immune complexes and polymorphonuclear neutrophils (11), as well as immunopathology mediated by T cells (8-10, 20, 28). Initial evidence supporting the latter ideal came from Metcalf's laboratory, which showed that athymic mice, unlike their euthymic littermates, failed to develop SK upon ocular infection with HSV (10). Subsequently, Russell and coworkers (20) confirmed the observations of Metcalf et al. and further implicated a role for T cells in SK by showing that the lesions occurred if athymic mice were reconstituted with T cells, in particular with T cells from HSV immune animals. Whether or not one or both major phenotypic subsets of T cells ($CD4^+$ and $CD8^+$) are involved in the immunopathological process remains ill defined. Accordingly, Russell et al. (20) initially suggested the involvement of class I major histocompatibility complex-restricted

cytotoxic T lymphocytes (CTL) in SK. This notion was later supported by Diefenbach and co-workers (14) on the basis of their studies with HSV mutants that were purportedly poor inducers of the CTL response and subsequently poor inducers of SK. Others have implicated the delayed type hypersensitivity (DTH) reaction, which in the case of HSV is mediated by CD4+ T cells (25-27), in SK immunopathology (8, 12, 13, 39). In the present report, we have readdressed the question of the role of one or the other of the two major phenotypic subsets of T lymphocytes in the immunopathology of SK by taking advantage of the recently available immunosurgical approach, in which mice can be selectively depleted of a given T cell subset by the *in vivo* administration of monoclonal antibody to their appropriate surface antigen (26, 32). Our results implicate the CD4+ cells as principally involved in the SK immune pathology. Moreover, we also demonstrate an essential role for CD8+ lymphocytes in controlling the severity of SK.

Chapter III

MATERIALS AND METHODS

Viruses

HSV typel (HSV-1) strains RE and KOS were grown on Vero cells supplemented with McCoy's 5A medium with10% heat inactivated donor calf serum (GIBCO Laboratories, Grand Island, N.Y.), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Recombinant vaccinia viruses containing the cloned genes for the influenza virus (A/JAP/305/57) hemagglutinin molecule (40), HSV-1 gB, gC,gD, alpha 0 and alpha 4 were grown in HEp-2 cells and purified as described previously (25). Virus was titered on Vero cells and the 50% tissue culture infective dose ($TCID_{50}$) per milliliter was determined at five days after infection. Virus was aliquoted in small amounts and stored at -70° C. A new aliquot of titered virus was thawed before use in subsequent experiments.

Mice

Male BALB/c AnNHsdBR and female C3H HenHsd (Harlan Spraque Dawley, Indianapolis, IN.) mice 6 to 8 weeks of age were used throughout the experiments. Surgical procedures were carried out by using the general inhalent anesthetic methoxyflurane (Pitman-Moore, Washington Crossing, NJ).

Corneal Inoculation

Inoculations on anaesthetized mice were achieved by proptosing the eye, and slightly abrading the cornea, in a crisscross pattern, with a 28g needle (Becton Dickinson and Company, Rutherford, NJ).

4µl of virus containing 10^6 $TCID_{50}$ was then placed on the corneal surface and gently massaged into the eye with the eyelids.

Monoclonal antibodies and T-cell subset depletion

The GK1.5 hybridoma was obtained from American Type Culture Collection, Rockville, MD. This cell line secretes rat monoclonal antibody reactive with the murine T cell surface antigen CD4 (L3T4), expressed on the helper-inducer T cell subsets (41). The 2.43 hybridoma was a generous gift of Dr. Frank Fitch (University of Chicago, Chicago, Illinois) and has been described previously (42). This cell line secretes rat monoclonal antibody reactive with the murine T cell surface antigen CD8 (Lyt 2.2), expressed on BALB/c T cells. The TIB 207 hybridoma was obtained from American Type Culture Collection, Rockville, MD. This cell line secretes rat monoclonal antibody reactive with Human HLA DR5 and was used as a rat IgG control.

Mice treated with anti-CD4, anti-CD8, anti-HLA or nonspecific mouse ascites (NMA) were inoculated intravenously on days -3, -2, and +2 with day 0 representing the day of HSV-1 corneal challenge. Mice were then reinoculated intraperitoneally with the same volume of antibody on days +7, +14, +21, and +28 to maintain the depleted state. The total amount of specific antibody in this volume of ascites, as determined by a modified enzyme-linked immunosorbent assay method, was 3 mg for anti-CD4, 0.1 mg for anti-CD8, and 0.3 mg for anti-HLA.

Flow cytometry

Cells from spleen and lymph nodes of individual mice were obtained at various times throughout the experiments, and incubated on ice for one hour with a 1/5000 dilution of heat-inactivated (56°C for 30 min) 50% ammonium sulfate-precipitated anti-CD4 or anti-CD8 antibodies diluted in RPMI 1640 media

containing 2% heat inactivated fetal bovine serum (GIBCO) and 0.2% sodium azide (Fisher Scientific Company, Fair Lawn, NJ). This was followed by three washes in the RPMI 1640 medium and second-step staining with fluorescein-conjugated anti-rat Ab (Southern Biotechnology Associates, Birmingham, AL). Cells were again washed three times and single-color fluorescence analysis was performed using a FACStar PLUS fluorescence-activated cell sorter (Becton-Dickinson).

Clinical and Pathological Observations

Severity of stromal keratitis was determined by observations with a slit lamp biomicroscope (Keeler Instruments, Broomall, Pa.) and based on the following scoring system: 0, no disease; 1+, slight corneal opacity; 2+, moderate opacity; 3+, severe opacity with the lens visible; 4+, opaque cornea; 5+, severe necrotizing keratitis. Scores were determined with the reader unaware of which mice belonged to which treatment group.

Tissues for light-microscope examination were fixed in 10% phosphate buffered formalin for 72 hours then embedded in paraffin wax. Twelve coronal sections per eye were then stained with hematoxylin and eosin (kindley performed by Jim Wesley).

Delayed Type Hypersensitivity assay

Five mice from each group were assayed for their ability to mount a DTH response. Mice were challenged by injecting a 50 µl solution, containing 1.5 x 10^6 $TCID_{50}$ of UV-inactivated HSV-1 or vaccinia virus, into the right rear footpad. The left rear footpads received a 50 µl solution containing UV-inactivated HEp-2 cell lysates. Mice whose corneas were abraded and which were inoculated with PBS served as a negative control. Footpad swelling was measured 24 hours after challenge with spring loaded calipers (Dyer Co., Lancaster, Pa.).

The amount of nonspecific swelling in the control left footpad was subtracted from that in the right rear footpad for final quantitation of the DTH response.

Antibody determinations

Serum collected from animals 38 days post infection was heat-inactivated (56°C for 20 minutes) and diluted in twofold dilutions, and for each dilution, 50 µl was aliquoted into 96 well flat-bottom microtiter plates (Costar, Cambridge, MA). To each well, 20 µl of medium containing 150 $TCID_{50}$ of the RE strain of HSV-1 was added, and the sealed plates were incubated at 4°C for 24 hrs. Each serum sample was represented in a minimum of six replicates. To one-half of the replicates, 50 µl of Low Tox rabbit complement (Cederlane Laboratories, Hornby, Ontario, Canada) diluted 1/4 was added, while the other replicates received 50 µl of media. Plates were then resealed and incubated at 37°C for one hour. Next, 10^4 Vero cells in a 50 µl solution were added to each well, and the plates were resealed and incubated for five days at 37°C. At four and five days later, wells were examined for cytopathic effect and the serum titers were determined as the reciprocal of the dilution that neutralized 50% of the virus, by the method of Spearman and Karber (43).

CTL assay

CTL cultures were generated by the method of Pfizenmaier et al. (44). Briefly, 5 days after the inoculation of 10^6 $TCID_{50}$ of HSV-1 KOS into the rear footpads, the draining popliteal lymph nodes were removed and single cell suspensions were prepared. Viable cells were adjusted to 2 x 10^6/ml in RPMI 1640, supplemented with 10% fetal bovine serum (heat inactivated at 56°C for 20 minutes), 7mM L-glutamine, 5 x 10^{-5} M 2-mercaptoethanol and cultured at 37°C as 20 µl aliquots in 100 mm^2 petri dishes (Becton

Dickinson Labware, Rutherford, N.J.). After 3 days of culture, cells were washed and viable cells were used as CTL effectors in a standard 4 hour chromium release assay as described previously (45).

Adoptive Cell Transfer into CD4 Depleted Mice

Mice were depleted of CD4+ T-lymphocytes by the *in vivo* administration of GK1.5. Ten days from the last antisera inoculation, and 72 hours after TC challenge, mice were given intravenous inoculations of spleen cells (5 x 10^7 cells) from donor mice which were previously subcutaneously immunized with 10^7 $TCID_{50}$ of HSV-1. This splenocyte source has been extensively characterized and consists predominantly of T-cells which express high levels of H-2 restricted cytotoxicity, contains helper cell activity, and can mediate delayed type hypersensitivity reactions (20).

Therapy using GK1.5

Mice were challenged via the TC route and subsequently placed into 4 different treatment groups. Group I was the control group and received anti-HLA on days +1, +2 and +3 post-infection, with day 0 representing the day of TC challenge. Group II received anti-CD4 prior to the clinical onset of SK; days +2, +4 and +6. Group III received anti-CD4 at the time of clinical onset of SK; days +6, +8 and +10. Group IV received anti-CD4 after the clinical onset of SK; days +12, +14, and +16. Mice were observed for clinical signs of the disease, using the slit lamp biomicroscope, for at least one month post-infection.

Protection experiments using vaccinia virus recombinants

Mice were immunized with 10^7 $TCID_{50}$ of the vaccinia recombinants described previously, and two weeks later were challenged via the TC route with the RE strain of HSV-1. Clinical observations of the ensuing SK were made for at least one month. Animals previously immunized with HSV served as the control group which were protected from a corneal challenge with HSV-1.

Chapter IV

Results

Phenotype of spleen and lymph node cells from mice treated with relevant antisera

To determine the effectiveness of antiserum treatment on depletion of T lymphocyte subsets, spleen and lymph node cells from test and control animals were analyzed for CD4+ and CD8+ cells by flow cytometry. The data in Fig. 1 (Appendix) are representative and depict profiles of spleen cells at 7 days following corneal challenge with HSV-1 and after 3 administrations of anti-T lymphocyte ascites or NMA. Typically, CD4+ cells were >95% depleted and CD8+ cells were >90% depleted. In the experiments, mice were given repeat injections of the relevant antisera on days 7, 14, 21, and 28 to maintain the depleted state. Profiles of test animals in various groups taken at the termination of experiments, on day 38, were similar to those shown in Fig. 1 (data not shown).

Effect of T cell depletion on the induction of DTH, production of neutralizing anti-HSV-1 antibody, CTL response, and central nervous system (CNS) disease

At 35 days post-infection, UV-inactivated HSV-1 was injected into one rear footpad of each of five mice from each of the treatment groups to test for DTH reactivity. As seen in Fig. 2, depletion of CD4+ bearing T cells significantly reduced the extent of virus specific DTH, whereas the depletion of CD8+ T cells had no effect upon the extent of DTH.

At 38 days postinfection, the mice described above were bled from the retro-orbital sinuses and the HSV-1 specific

antibody response was determined. The data shown in Table 1 demonstrate that whereas CD8+ T cell depletion had no effect upon the levels of antiviral antibodies, mice depleted of CD4+ T cells produced either marginal or undetectable anti-HSV-1 neutralizing antibody. In accordance with previous reports (26), the *in vivo* depletion of CD4+ bearing T lymphocytes prior to the inoculation of HSV-1 did not abrogate the subsequent development of HSV-1 specific CTL (fig. 3). Conversely, the HSV-1 specific CTL response was completely inhibited in CD8+ depleted mice (fig 4).

An interesting observation was that although mice depleted of CD4+ T lymphocytes subsequently lost the ability to mount a significant DTH or neutralizing antibody response, all animals remained healthy throughout the HSV-1 challenge experiments. In contrast, in some experiments, mice depleted of CD8+ T lymphocytes lost weight, had hind limb paralysis, and subsequently died within 2 weeks of initiation of the experiment (fig. 5).

Comparison of corneal stromal disease in intact and CD4+ and CD8+ depleted mice

Mice were examined for SK by slit lamp biomicroscopy approximately every 2 days after infection. Epithelial damage was observed by 2 days post-infection in all mice inoculated with HSV-1. This was normally followed by a mild inflammatory reaction which resolved soon thereafter. Peripheral corneal neovascularization and stromal infiltration were evident within approximately one week in those mice which developed SK. All mice in the NMA control group and the CD8+ depleted group developed various stages of SK within 3 weeks post-infection and the majority of these animals developed the more intense forms of SK. In contrast, throughout the entire experiment a maximum of 30% of the mice in the CD4+ depleted group developed SK. The

initiation of SK in CD8+ depleted mice was comparable to that in the NMA group, but the disease in the CD8+ depleted group was more severe and developed more rapidly (Table 2). Random mice were chosen from each group at the termination of the experiments, and eyes were taken for histological examination. The corneas from mice treated with NMA, as well as from control mice, were heavily infiltrated with both mononuclear and polymorphonuclear leukocytes. However, corneas from CD4+ depleted mice showed only mild inflammatory responses. In the CD8+ depleted mice, corneal inflammatory responses were similar to those of the control group. On the basis of the severity of SK in this treatment group (as determined by slit lamp microscopy), we expected a more intense infiltration in these mice, but this was not evident as judged histologically.

In Fig. 6 and 7 a compilation of the average severity of SK for all mice in each treatment groups is presented. The results indicate that removal of CD4+ T cells markedly reduced the immunopathology associated with herpetic SK, whereas removal of T cells expressing the CD8+ phenotype resulted in an enhanced severity of SK.

Effect of reconstituting CD4+ depleted animals with HSV-1 immune splenocytes

Mice were depleted of CD4+ T-lymphocytes by the in vivo administration of GK1.5. Ten days following the last antisera treatment and 72 hours post-infection, these animals were reconstituted with T-lymphocytes via an intravenous inoculation of splenocytes from HSV-1 donor mice. This splenocyte source has been extensively characterized and consists predominantly of T-cells which express high levels of H-2 restricted cytotoxicity, contains helper cell activity, and can mediate delayed type hypersensitivity reactions (20). The effect this reconstitution had on the development of SK was then evaluated by slit lamp

biomicroscopy. The results of these experiments indicated that the majority of normal (anti-HLA treated) mice developed significant levels of SK, while mice depleted of CD4 had significantly reduced levels of SK. As seen in figure 8 mice which were depleted of CD4+ T-lymphocytes and subsequently reconstituted with HSV-1 immune splenocytes developed, on average, levels of SK which were intermediate to the normal and CD4 depleted animals. These results provide further evidence for the essential role of T-lymphocytes in mediating the stromal reaction.

Therapy of SK using GK1.5

Mice were challenged via the TC route and subsequently placed into 4 different treatment groups. Group I was the control group and received anti-HLA on days +1, +2 and +3 post-infection, with day 0 representing the day of TC challenge. Group II received anti-CD4 prior to the clinical onset of SK; days +2, +4 and +6. Group III received anti-CD4 at the time of clinical onset of SK; days +6, +8 and +10. Group IV received anti-CD4 following the clinical onset of SK; days +12, +14, and +16. As shown in figure 9, it appears that the timing of anti-CD4 administration is important in the treatment of SK. Thus, when anti-CD4 is administered prior to or during the onset of disease then the resultant immunopathology was abrogated. However, once the stromal reaction is underway then there is no significant reduction in the severity of SK following anti-CD4 therapy.

Attempts to define the specificity of the immunopathological CD4+ T-lymphocytes

In an effort to determine which HSV-1 antigen(s) induce the CD4+ T-lymphocytes to mediate the immunopathology, mice were immunized with a variety of vaccinia virus recombinants which

express HSV-1 structural or non-structural proteins, and then challenged via the TC route. The effect of these immunizations on the subsequent development of disease was then determined by slit-lamp examinations. The results of these experiments are shown in figures 10 and 11 and they indicate that protection from SK was achieved by the prior immunization of vaccinia recombinants which express HSV-1 structural glycoproteins. However, immunizations with the tested non-structural protein expressing recombinants did not confer protection to any animals. Since it is assumed that the protection conferred by recombinants in the former group is mediated by T-lymphocytes, then splenocytes from these animals will be used in future experiments to reconstitute CD4 depleted animals in an attempt to define the target antigen(s) recognized by the immunopathological CD4+ T-lymphocytes.

Chapter V

DISCUSSION

In most circumstances, disease associated with HSV infection results from cytolytic effects of the virus and therapy with antiviral drugs represents a valuable approach. However, in a few situations, immunopathological responses to persisting virus or viral antigens account for the lesions and strategies for therapeutic intervention must differ. By far the most significant syndrome which is assumed to result from an immunopathological response to HSV is SK, a disease that is among the most common causes of visual impairment in the United States (39). Our present report provides evidence for the immunopathological nature of SK and further demonstrates that T cells of the CD4+ phenotype play an essential role in the process. Accordingly, whereas infection of nondepleted animals with HSV-1 via the cornea was followed by stromal lesions, lesions were absent or markedly diminished in mice depleted of CD4+ lymphocytes by the *in vivo* administration of anti-CD4 serum. In fact, although the majority of nondepleted mice developed severe SK, only a minor proportion of the mice in the CD4+ depleted group developed SK, and in these mice the lesions were less severe in nature. Presumably, these lesions evident in some CD4+ depleted mice were accounted for by the approximately 5% of CD4+ cells that remain after the *in vivo* antiserum therapy. However, it is possible that more than one mechanism of immunopathology could account for the SK lesions, as has been discussed earlier (39). Some have suggested that SK could represent an immune-complex lesion with polymorphonuclear leukocytes principally involved in the inflammatory response (11). Our results cannot exclude a role for toxic immune complexes, but we favor the hypothesis that the

mechanism by which the stromal reaction is carried out is by means of CD4+ cells that mediate a DTH reaction (25, 27). Thus CD4+ suppressed mice failed to produce a DTH response to HSV, and previous observations had demonstrated that the inability of athymic mice to generate SK could be reconstituted by adoptive transfers of T cells capable of mediating DTH (46). The results of experiments on split tolerance also substantiate an essential role for DTH in SK (discussed subsequently), and the essentially mononuclear cell nature of the stromal reactions resembles that of DTH rather than immune-complex lesions. Of course SK may also involve a variety of other immune cells working simultaneously to destroy the integrety of the eye. For example antibody produced by B-cells in response to HSV may attach to virally infected stromal keratocytes and initiate the cascading events of complement with the end result of this process leading to lysis and eventual destruction of the infected cells. In fact, enzymes normally produced in stromal keratocytes may be released upon the destruction of the cell and this may contribute to additional toxicity and damage within the corneal stroma.

Although no one has shown a role for natural killer (NK) cells in SK, a possible role for this immune cell can be envisioned. NK cells have been shown to lyse virally infected cells *in vitro* , therefore damaging effects of these cells within the corneal stroma cannot be ruled out.

Indirect tissue damage mediated by macrophages or PMN is yet another possibility. Macrophages are crucial to the initiation of immune responses via there ability to present antigens and PMN are able to secrete chemicals which function in the effector phase of the immune response. For example, a variety of anti-microbial chemicals are released by macrophages and PMN following the recognition of a particular pathogen. These include, among others; hydrogen peroxide, superoxide radicals, acid hydrolases and cationic proteins, all of which may potentially be toxic to stromal keratocytes. As mentioned previously the

depletion of PMN from animals challenged via the TC route spared them of the damaging effects of SK. Regardless of the actual mechanism(s) of immunopathology, this communication supports an essential requirement for CD4+ T-lymphocytes either in the initiation or propagation of the disease. The detailed role for this T-lymphocyte can only be speculated. A likely mechanism is however, that upon invasion of HSV into the cornea, macrophages, and other phagocytic cells ingest the virus, degrade it and present it to B and T-lymphocytes. While the lymphocytes are undergoing clonal expansion, macrophages may secrete anti-microbial chemicals at the site of infection, and a high concentration of these chemicals may cause toxic damage to the stromal keratocytes. Once the CD4+ T-lymphocytes have been recruited to the site of infection, it is likely that they release lymphokines which activate, recruit and retain other B and T-lymphocytes. This reaction is similar to, if not indicative of a DTH response. Thus, it is likely at this stage of the disease that toxic immune complexes and CTL, in conjunction with high concentrations of lymphokines, and tissue enzymes, cause the majority of tissue damage characteristic of SK. This communication also supports a requirement for CD8+ T-lymphocytes in down-regulating the immune response, likely by there known suppressor activity. Thus, the immunopathology of CD8+ depleted animals occurs more rapidly and these animals are more susceptible to the severe forms of SK.

Immunopathology in SK resulting from a cytotoxic T lymphocyte mediated event has also been suggested (20). Support came from the results of adoptive transfer experiments, as well from the observation that gC mutant HSV viruses that failed to act as CTL targets were also incapable of causing stromal reactions following corneal infection of AJ (H-2^a) mice. A follow up to this study (29) by the same investigators employed the use of HSV-1 mutant viruses which did not express glycoprotein C (gC-). According to this report, upon AC injection of gC- virus followed by TC inoculation of wild-type virus, mice undergo a

unique set of immune responses, whereby the mice are tolerized for a CTL response, but not a DTH response. This report also claims that these animals do not undergo SK reactions therefore, a role for DTH in the immunopathology can be ruled out, and the likely mediators of immunopathology are CTL. What this report fails to address however, is why this is the first report ever, whereby a particular antigen has been placed into the anterior chamber of an eye and not tolerized for a DTH response. Furthermore, this report did not address why the same toleragenic effect on DTH was not seen when using a one amino acid substitution in an additional gC- virus. These results are unexpected when one takes into account the knowledge that this particular amino acid substitution would likely not have affected the three dimensional conformation of the peptide which would have induced the immune response.

Our present report, however, provides no support for a role of CD8+ major histocompatibility complex class I restricted CTL in the immunopathological reaction of BALB/c (H-2^d) mice. Accordingly, vigorous SK reactions still occurred in mice depleted of CD8+ lymphocytes. These mice were also determined to be markedly diminished in their HSV CTL responsiveness. Indeed, although the onset of SK in CD8+ depleted mice was similar to that in control mice, its severity was, on the average, increased in these animals. After a period of 28 days, virtually all mice in the latter group developed symptoms of CNS disease. Consequently, it appears that rather than mediating immunopathology, CD8+ cells perform a protective role. How such a role is mediated needs definition, but on the basis of previous reports by others (47, 48) as well as ourselves (24), we favor the idea that the protective effect by CD8+ cells is mediated at least in part by their known suppressor cell activity, which in this instance, is acting against the CD4+ mediated DTH response. A similar protective effect was demonstrated by Altmann and Blyth (49) by using a model in which an immunopathological reaction against HSV occurred in

the CNS which appeared on the basis of adoptive transfers, to be mediated by a DTH reaction that caused demyelination of sensory nerves. In that study, they demonstrated that suppression of the HSV-DTH response by T suppressor cells inhibited the immunopathological damage. Many others have also demonstrated that the HSV specific DTH reaction is subject to modulation by suppressor cells at either the afferent or efferent level (50, 51). In our CD8+ depleted mice, we could still demonstrate potent DTH reactions although, perhaps unexpectedly, therse were not increased in magnitude over those observed in nonsuppressed mice. However, subtle increases in activity might be observed in CD8+ depleted mice if measured by more analytical assays. We are currently attempting to perfom such analysis by using an adoptive transfer approach.

The importance of confiming the nature of the pathogenesis of SK and of establishing the mechanism of its immunopathology lies with the ultimate strategy chosen for treatment and prevention of this important disease. Consequently, since CD4+ and CD8+ cells show different restriction specificities and appear to respond to different fragmentation products of target proteins (52), any vaccine designed to stimulate or suppress these subsets must take such facts into consideration. In addition, strategies are known in which the immune response induced may differ in terms of the array of immunological activities generated. Thus, in the HSV system, several approaches result in a negative DTH reaction but a positive response in all other measured aspects of immunity. This so-called split tolerance can be produced either by immunizing mice with virus by the intravenous (22), intraperitoneal (21), or intracameral (23) route or by exposure of mice to certain anti-idiotype reagents (24). In this latter circumstance, we have demonstrated that exposure of mice to a heterologous anti-idiotype serum produced against a monoclonal anti-glycoprotein C antibody causes a suppressor cell mediated inhibition of the DTH reaction to HSV. Moreover, the use of such

an anti-idiotype reagent in mice renders the suppressed in their ability to generate an immunopathological SK following corneal infection with HSV. The use of the anti-idiotype reagent, as well as other means of selectively modulating CD4 cell function, is currently under investigation as a means of modulating the SK reaction against HSV.

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APPENDIX

Figure 1. In vivo depletion of T cell subsets after anti-CD4 or anti-CD8 treatment. Cells from spleens of individual mice which received antiserum treatment on days -3, -2, and +2 (with day 0 representing the day of HSV-1 corneal challenge) were obtained on day 7. Cells were then stained and analyzed for the presence of CD4+ and CD8+ T lymphocytes by flow cytometry. (A) Normal CD4+ population of T lymphocytes from mice treated with anti-CD8 monoclonal antibody. (B) Depletion of the CD8+ T lymphocyte subset following anti-CD8 treatment. (C) Depletion of the CD4+ T lymphocyte subsets following anti-CD4 treatment. (D) Normal CD8+ population of T lymphocytes from mice treated with anti-CD4. (E) Normal CD4+ population of T lymphocytes from mice treated with NMA. (F) Normal CD8+ population of T lymphocytes from mice treated with NMA.

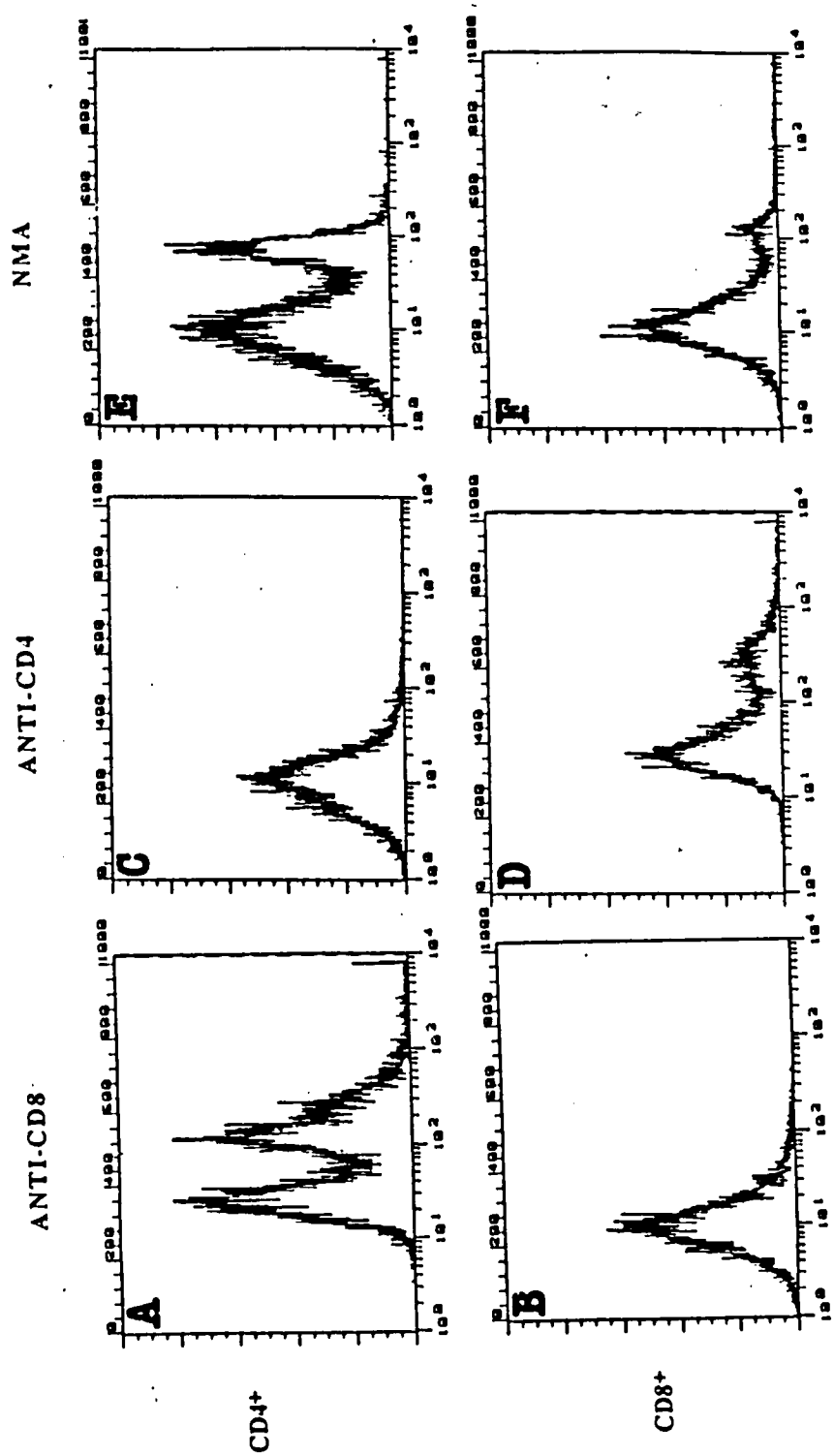

Figure 1.

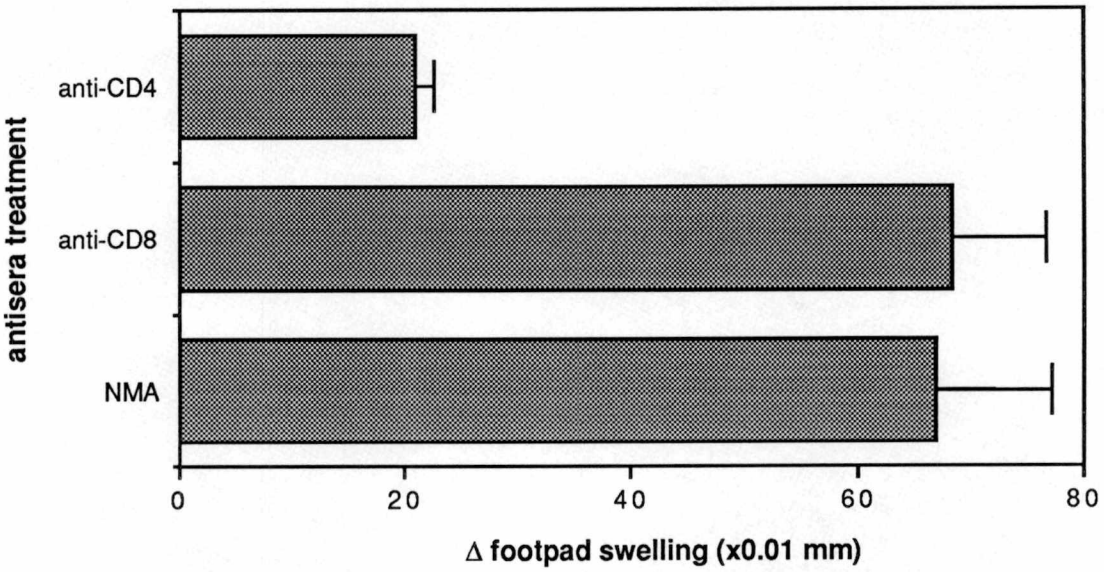

Figure 2. Effect of in vivo T cell depletion on the generation of an HSV-specific DTH response. At 35 days post infection, five mice from each treatment group were challenged in the right rear footpad with 1.5 x 10^6 $TCID_{50}$ of UV-inactivated virus. The left rear footpads received an equal volume of HEp-2 cell lysate. Footpad swellings were measured with spring loaded calipers at 24 hours after challenge. The amount of background swelling of the control left footpad was subtracted from the swelling observed in the test right footpad. Data are expressed as the mean of results from five mice plus or minus one standard error. For anti-CD4 versus NMA, $P < 0.005$.

Figure 3. HSV-1 specific CTL activity in intact and CD4+ depleted mice. Lymphocytes which were either mock (NMA) or CD4+ (GK1.5) depleted were obtained from the popliteal lymph nodes of acutely infected C3H HenHsd mice and were cultured as described previously and used as a source of CTL effectors in a 4 hour chromium release assay. Target cells were infected with the respective viruses shown (multiplicity of infection, 5) and labeled with $Na_2{}^{51}CrO_4$ (200μCi) for 4 hours prior to the assay. Target cells were autologous L929 cells ($H\text{-}2^k$) or allogeneic A31 cells ($H\text{-}2^d$). CTL populations were used at an effector to target ratio of 30:1 and percent specific lysis was determined as described previously (13). Results are shown as individual values of 16 NMA (a) and 12 CD4 depleted (b) replicate cultures, with the broken lines representing the mean plus or minus one standard deviation.

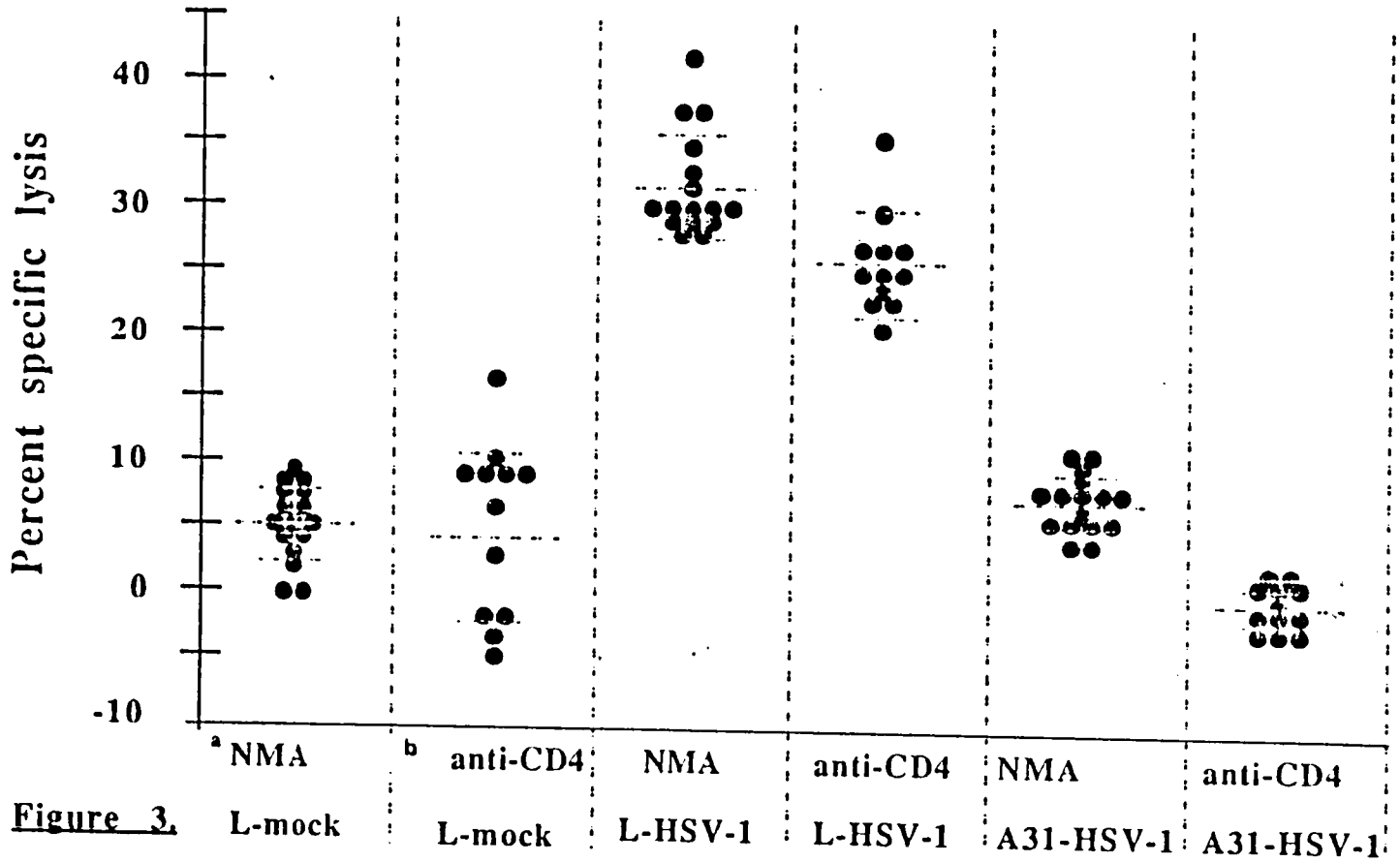
Percent specific lysis
40
30
20
10
0
-10
[a] NMA
L-mock
[b] anti-CD4
L-mock
NMA
L-HSV-1
anti-CD4
L-HSV-1
NMA
A31-HSV-1
anti-CD4
A31-HSV-1

Figure 3.

Figure 4. Loss of HSV-1 specific CTL activity in CD8+ depleted mice. Lymphocytes which were either mock (NMA) or CD8+ (2.43) depleted were obtained from the popliteal lymph nodes of acutely infected BALB/c mice and were cultured as described previously as a source of CTL effectors in a 4 hour chromium release assay, as described in the legend to figure 3. Target cells were autologous EMT6 cells ($H\text{-}2^d$) or allogeneic L929 ($H\text{-}2^k$). CTL populations were used at an effector to target ratio of 50:1. Results are shown as individual values of 12 replicate cultures, with the broken lines representing the mean plus or minus one standard deviation.

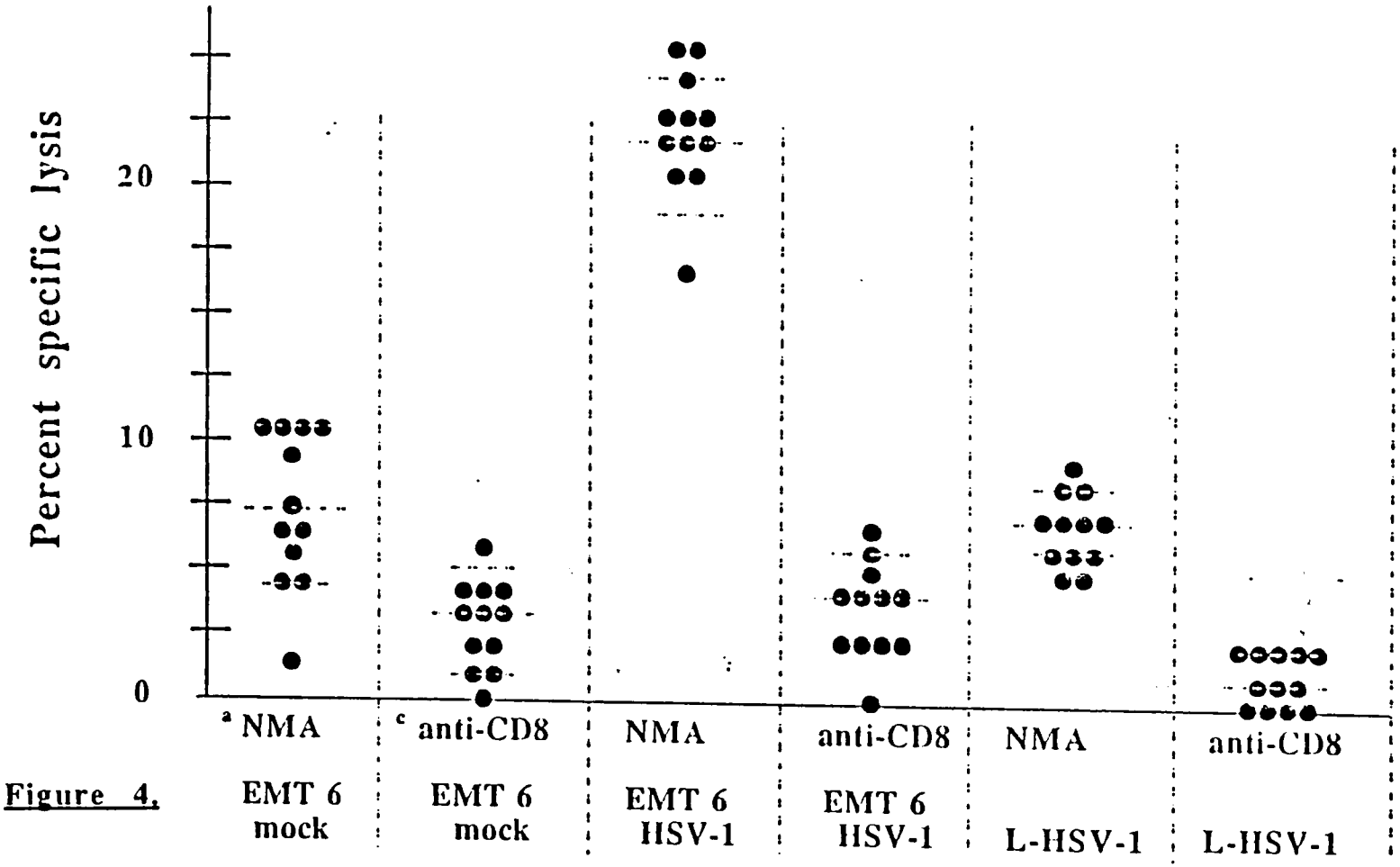
Percent specific lysis
20
10
0
[a] NMA
[c] anti-CD8
NMA
anti-CD8
NMA
anti-CD8
EMT 6 mock
EMT 6 mock
EMT 6 HSV-1
EMT 6 HSV-1
L-HSV-1
L-HSV-1

Figure 4.

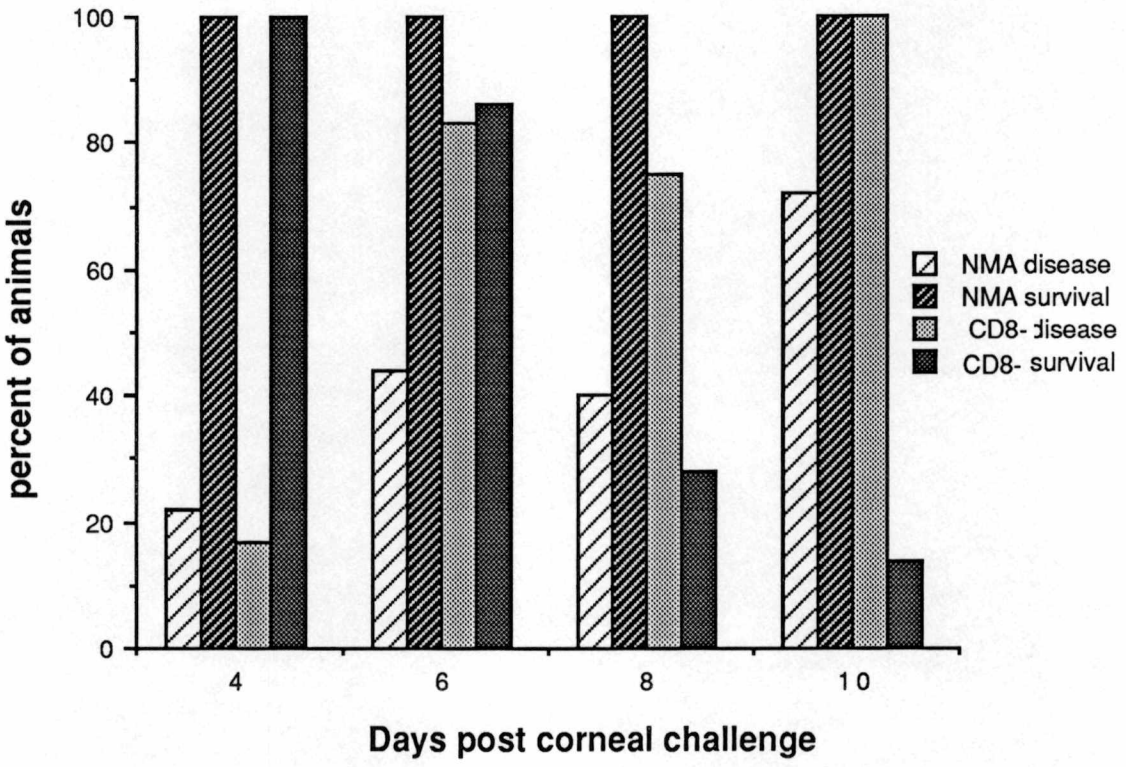

Figure 5. Susceptibility of CD8+ T cell depleted mice to CNS disease following HSV-1 corneal challenge. BALB/c mice were inoculated with 0.100 mg of anti-CD8 (2.43) monoclonal antibodies or nonspecific mouse ascites on days -3, -2, and +2. Corneas were abraded in a checkerboard configuration with a 28 gauge needle prior to topical corneal inoculation with 10^6 $TCID_{50}$ of the RE strain of HSV-1 on day 0. Mice were then inoculated on day 7 with the monoclonal antibody to prolong the depleted state. In some experiments, during examination for stromal disease, animals displayed signs of CNS disease such as weight loss, hind-limb paralysis, and eventually death.

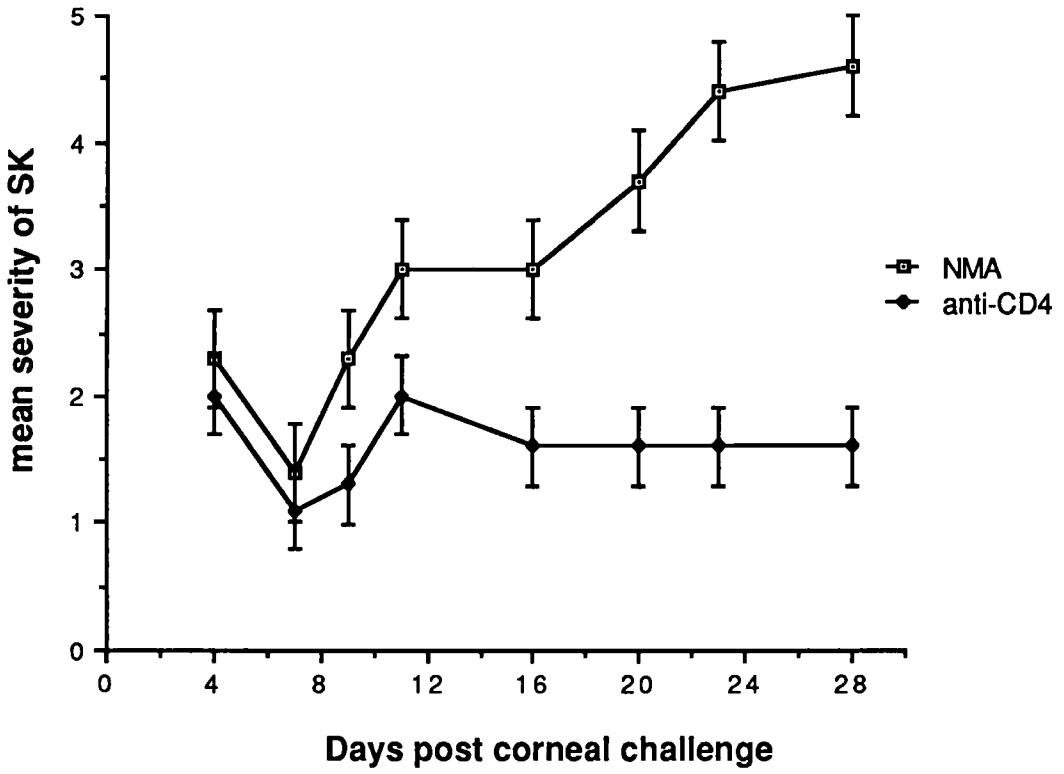

Figure 6. Therapy with anti-CD4 reduced the severity of stromal keratitis. BALB/c mice were inoculated with 3 mg of anti-CD4 (GK1.5) monoclonal antibody or nonspecific mouse ascites on days -3, -2, and +2. Corneas were abraded in a checkerboard configuration with a 28 guage needle prior to topical corneal inoculation with 10^6 $TCID_{50}$ of the RE strain of HSV-1 on day 0. Mice were then inoculated once per week with the monoclonal antibody to prolong the depleted state. Eyes were examined for stromal disease with a slit lamp biomicroscope, and scores were determined as previously described. Day 20 anti-CD4 versus NMA, $P < 0.01$; day 23 anti-CD4 versus NMA, $P<0.005$.

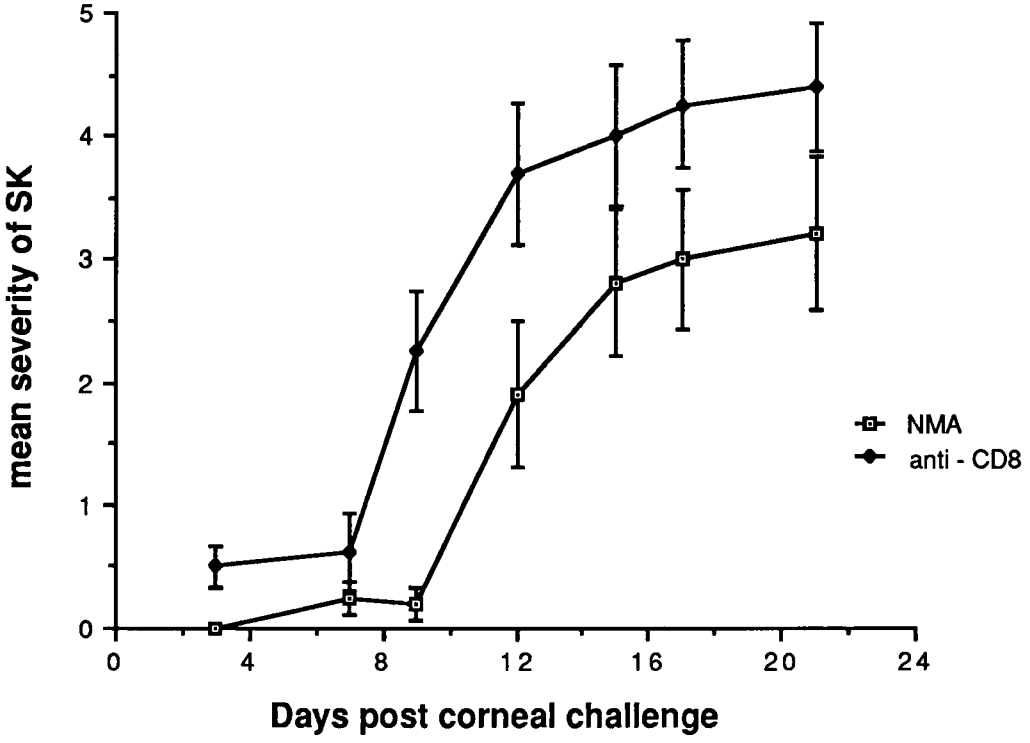

Figure 7. Therapy with anti-CD8 increased the severity of stromal keratitis. BALB/c mice were inoculated with 0.100 mg or anti-CD8 (2.43) monoclonal antibody or nonspecific mouse ascites on days -3, -2, and +2. Corneas were abraded in a checkerboard configuration with a 28 guage needle prior to topical corneal inoculation with 10^6 $TCID_{50}$ of the RE strain of HSV-1 on day 0. Mice were then inoculated once per week with the monoclonal antibody to prolong the depleted state. Eyes were examined for stromal disease with a slit lamp biomicroscope, and scores were determined as previously described. Day 9 anti-CD8 versus NMA, $P < 0.005$; day 12 anti-CD8 versus NMA, $P < 0.05$.

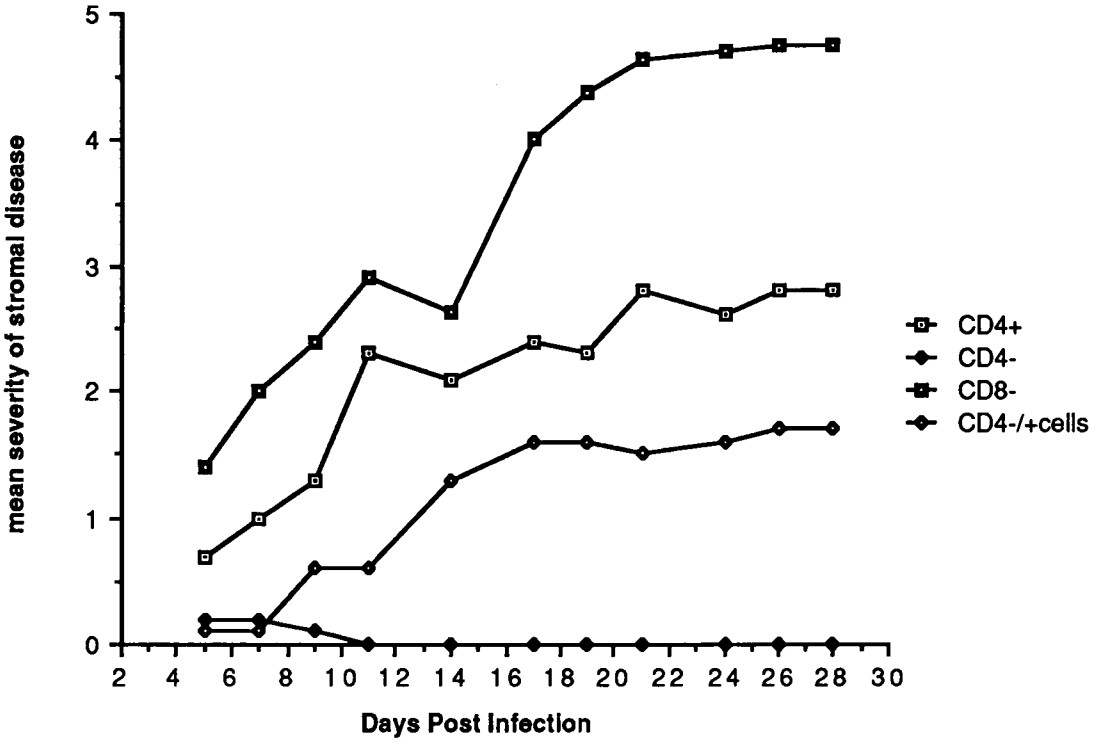

Figure 8. Adoptive transfer of HSV-1 immune splenocytes can reconstitute SK in CD4- mice. Mice were depleted of CD4+ T-lymphocytes by the in vivo administration of GK1.5. Ten days following the last antisera inoculation, and 72 hours after TC challenge, CD4- mice were given intravenous inoculations of spleen cells (CD4-/+cells) from donor mice which were previously immunized with 10^7 $TCID_{50}$ of HSV-1. The ability to reconstitute disease in CD4- animals (CD4-) was then assessed by slit lamp biomicroscopy. Normal mice (CD4+) and CD8 depleted animals (CD8-) served as additional controls.

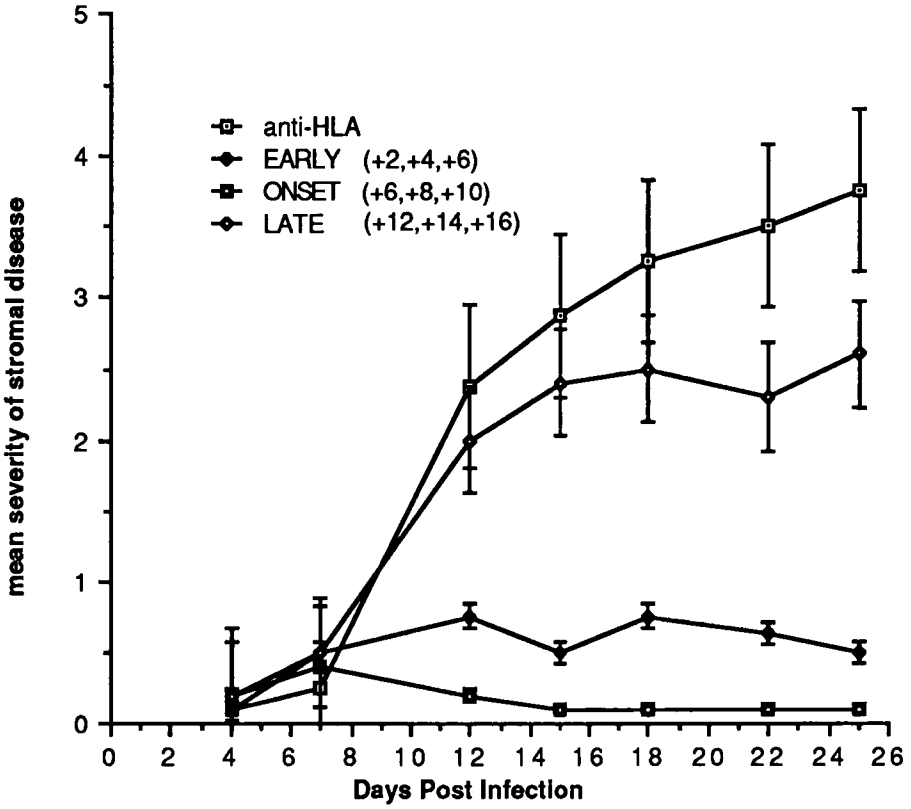

Figure 9. Early therapy with anti-CD4 antibody can reduce the severity of SK. Mice were challenged via the TC route and subsequently placed into 4 different treatment groups. Group I was the control group and received anti-HLA on days +1, +2 and +3 post-infection, with day 0 representing the day of TC challenge. Group II received anti-CD4 prior to the clinical onset of SK; days +2, +4 and +6. Group III received anti-CD4 at the time of clinical onset of SK; days +6, +8 and +10. Group IV received anti-CD4 after the clinical onset of SK; days +12, +14, and +16. Mice were observed for clinical signs of the disease using the slit lamp biomicroscope.

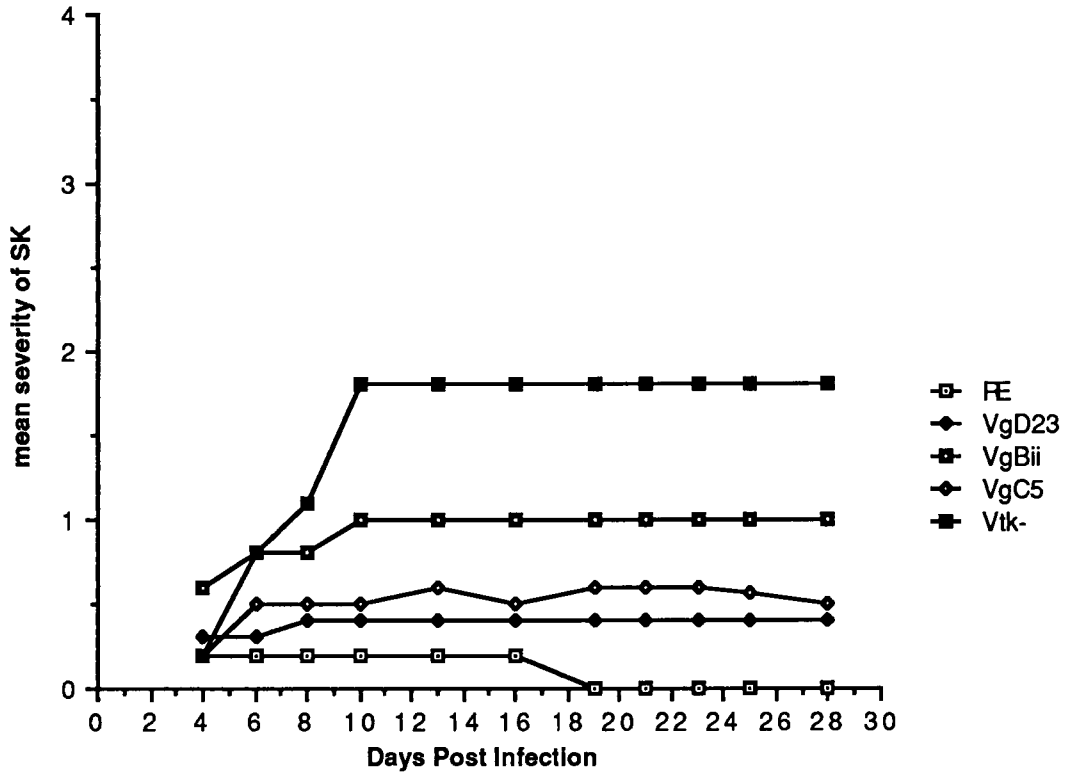

Figure 10. Mice are protected from SK by prior immunization with vaccinia recombinants expressing HSV-1 structural glycoproteins. Mice were immunized with 10^7 $TCID_{50}$ of vaccinia recombinants expressing HSV-1 structural glycoproteins, and two weeks later were challenged via the TC route with the RE strain of HSV-1. Clinical observations of the ensuing SK were made for at least one month. The recombinants expressed HSV structural glycoproteins; B (VgBii), C (VgC5), and D (VgD23). HSV immune animals (RE) and animals immunized with vaccinia virus which express the influenza hemagglutinin molecule (Vtk-) served as positive and negative controls respectively.

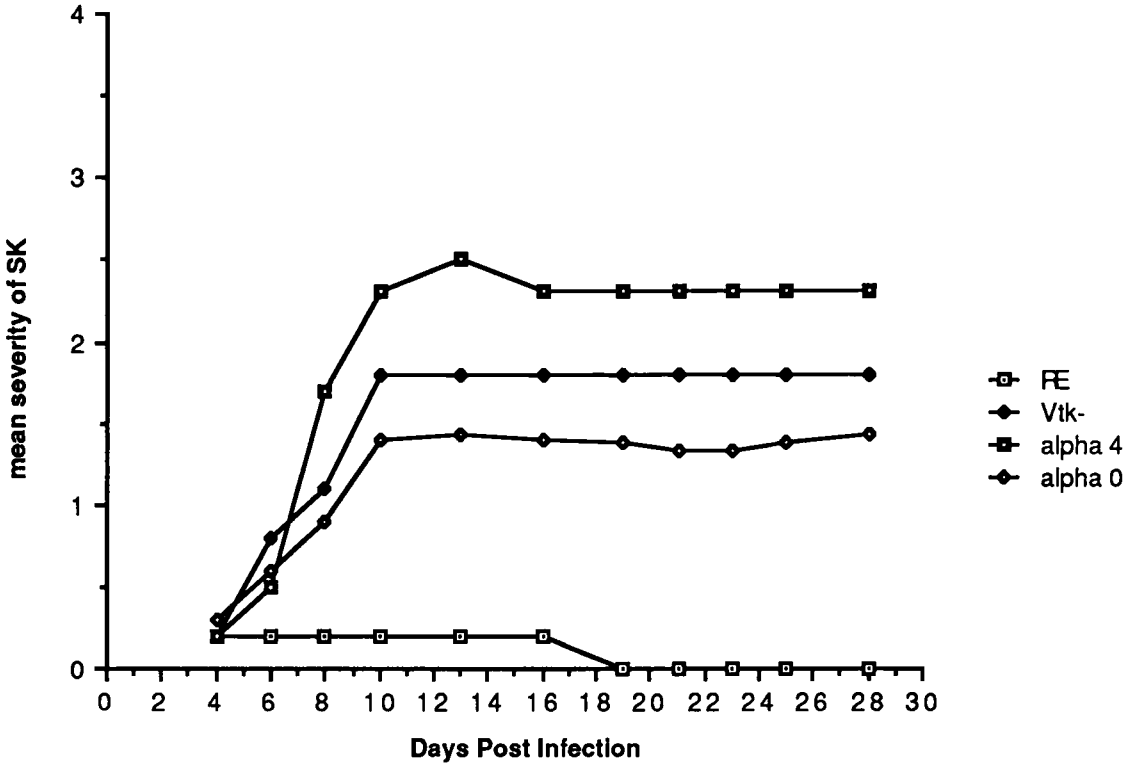

Figure 11. Mice are not protected from SK by prior immunization with vaccinia recombinants expressing HSV-1 non-structural glycoproteins. Mice were immunized with 10^7 $TCID_{50}$ of vaccinia recombinants expressing HSV-1 non-structural proteins, and two weeks later were challenged via the TC route with the RE strain of HSV-1. Clinical observations of the ensuing SK were made for at least one month. The recombinants expressed HSV non-structural proteins; alpha 0 and alpha 4. HSV immune animals (RE) and animals immunized with vaccinia virus which express the influenza hemagglutinin molecule (Vtk-) served as positive and negative controls respectively.

TABLE 1: Levels of Neutralizing Antibodies in Intact and T Cell Subset Depleted Mice

Antisera Treatment	Inverse of Neutralization Titers[a]	
	- Complement	+ Complement
Anti-CD4	<5	54 ± 7[b]
Anti-CD8	25 ± 0.5	2284 ± 497
Nonspecific Mouse Ascites	37 ± 9	2980 ± 639

Figure 12. Levels of neutralizing antibodies in intact and T cell subset depleted mice. [a] Titers were measured at 38 days after corneal challenge with HSV-1 and are expressed as means plus or minus the standard error of five mice per group. Five preimmune mice served as a negative control, and their neutralization titers were below the detection limits of the assay. Serum collected from mice immunized subcutaneously with HSV-1 had neutralization titers of 35 without (-) complement and greater than 7000 with (+) complement.

[b] Significantly less than NMA control ($P<0.005$).

Figure 13. Development of HSV stromal keratitis after the in vivo depletion of T cell subsets. [a] Severity of SK was determined by observations with a slit lamp biomicroscope and based on the following scoring system: 0, no disease; 1+, slight corneal opacity; 2+, moderate opacity; 3+, severe opacity with the lens visible; 4+, opaque cornea; 5+, severe necrotizing keratitis.

Days Post Infection	Severity[a,b]	Antisera Therapy: Nonspecific Mouse Ascites	Anti-CD8	Anti-CD4
	0	8/10 (80%)	9/16 (56%)	9/10 (90%)
	+1	2/10 (20%)	6/16 (38%)	1/10 (10%)
	+2	0/0 (0%)	0/0 (0%)	0/0 (0%)
Day 7	+3	0/0 (0%)	1/16 (6%)	0/0 (0%)
	+4	0/0 (0%)	0/0 (0%)	0/0 (0%)
	+5	0/0 (0%)	0/0 (0%)	0/0 (0%)
	0	1/10 (10%)	2/16 (12.5%)	7/10 (70%)
	+1	2/10 (20%)	0/0 (0%)	0/0 (0%)
	+2	3/10 (30%)	2/16 (12.5%)	0/0 (0%)
Day 14	+3	0/0 (0%)	0/0 (0%)	3/10 (30%)
	+4	0/0 (0%)	0/0 (0%)	0/0 (0%)
	+5	4/10 (40%)	12/16 (75%)	0/0 (0%)
	0	1/10 (10%)	1/16 (6.3%)	7/10 (70%)
	+1	2/10 (20%)	2/16 (12.5%)	0/0 (0%)
	+2	1/10 (10%)	0/0 (0%)	0/0 (0%)
Day 21	+3	0/0 (0%)	0/0 (0%)	0/0 (0%)
	+4	1/10 (10%)	0/0 (0%)	3/10 (30%)
	+5	5/10 (50%)	13/16 (81.3%)	0/0 (0%)
	0	2/10 (20%)	1/16 (6%)	7/10 (70%)
	+1	1/20 (10%)	0/0 (0%)	0/0 (0%)
	+2	1/10 (10%)	0/0 (0%)	0/0 (0%)
Day 28	+3	0/0 (0%)	0/0 (0%)	0/0 (0%)
	+4	0/0 (0%)	0/0 (0%)	2/10 (20%)
	+5	6/10 (60%)	15/16 (94%)	1/10 (10%)

VITA

Charles K. Newell was born on July 13, 1962, in Panama City, Florida, home of the "Worlds Most Beautiful Beaches." He attended Beach elementary school and graduated from Bay County High School, in Panama City in June 1980. After a one year academic hiatus he enrolled in Florida State University, where his promising career as a football jock was cut short. Turning instead to academics, he received a Bachelor of Science degree in Biology in 1985.

Following one year as a research associate with Dr. Barry T. Rouse he was accepted into the department of Microbiology Graduate program at the University of Tennessee, in 1986. He was awarded the Master of Science degree in Microbiology in May 1989.

The author is uncertain of his future, but is resolved to make it a successful one.