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The involvement of T lymphocyte subsets in the immunopathology of herpetic stromal keratitis (HSK)

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To the Graduate Council:

I am submitting herewith a dissertation written by Mehmet Ziya Doymaz entitled "The involvement of T lymphocyte subsets in the immunopathology of herpetic stromal keratitis (HSK)." I have examined the final electronic 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:

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Dr. Barry T. Rouse, DVM, Ph. D. Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

THE INVOLVEMENT OF T LYMPHOCYTE SUBSETS IN THE IMMUNOPATHOLOGY OF HERPETIC STROMAL KERATITIS (HSK)

A dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Mehmet Ziya Doymaz

August 1991

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PART I:

LITERATURE REVIEW

1. INTRODUCTION

The term immunopathology (IP) is used to describe pathological conditions which are caused by different effector arms of the immune system such as cytotoxic T cells, hypersensitivity responses, and antibodies (Sell, 1987).The end product of immunopathological reactions are functional impairments and morphological degenerations in the affected tissues. The characteristic feature of immunopathological reactions is an unwanted and uncontrolled immune response. Implicit in this feature is that experimental manipulations on the functions of the immune elements result in dramatic changes in the clinical symptoms of immunopathological conditions. Thus, immunosuppressive, or immunopotentiating regimens have been the method of choice to study immunopathological models. As more sophisticated means of manipulating immune effector cells have become available, our detailed understanding of the mechanisms of IP have increased considerably (Kumar et.al., 1989). In classical immunology textbooks, immunopathological reactions are classified according to:

-the mechanisms of the pathology e.g. allergic reactions, and immune complex reactions,

-the source of the immune factors mediating the IP e.g. endogeneous immune factor mediated (autoimmune type reactions), and exogenous immune factor mediated (graft versus host reactions),

-the source of the antigen to which immunopathological responses are directed e.g. exogenous antigens (tuberculin reactions), and endogenous antigens (demyelinating diseases).

In the first half of this review, the IP of viral infections, and the role of individual T lymphocyte subsets in the development of these conditions will be outlined. The discussion will be limited to selected viral and parasitic models where the pathology is mediated by T lymphocytes and an adoptive transfer approach has been established for IP. At times, the length of this section seems exhaustive, however, many of the conclusions drawn from these models are crucial in understanding and evaluating the current status and future directions of investigations on herpetic stromal keratitis (HSK). The second half of the

review will summarize immunopathological conditions observed in herpes simplex virus (HSV) infections with an emphasis on herpetic stromal keratitis (HSK).

2. IMMUNOPATHOLOGY IN VIRUS INFECTIONS

Viral infections have diverse immunopathological consequences.The diversity extends to both the mechanisms of immunopathology and the tissues which are affected (Sissons and Borysiewicz, 1985). However, several generalizations about viral IP can be made. Firstly, virus infection itself serves usually as a triggering event in the pathology (Schattner and Rager-Zisman, 1990). Subsequently, an uncontrolled immune response is mounted to either viral antigens or autoantigens which, under normal conditions, are not recognized by the immune system. In the absence of immunopathological responses, virus is either eliminated from the body by other less costly defensive means without harmful consequences or viral presence in the tissues does not cause any functional impairment (Schattner and Rager-Zisman, 1990).

Depending upon the arm of the immune system causing the damage, immunopathological conditions can be potentiated by the two arms of the immune system: humoral immune response-mediated, and T cell-mediated IP.

2.1. The role of humoral immune responses in the development of immunopathology

The best examples of humorally-mediated IP are immune complex (IC) diseases. The critical parameter in the formation of IC diseases is the production of low affinity antibodies or antibodies specific for non-critical epitopes of the invading pathogen (Mims, 1983). Antibodies complexed with antigen normally are cleared from the circulation by variety of cells. This process results, in most cases, in the complete elimination of the antigens. In IC diseases, however, ineffective elimination of IC causes the activation of harmful mediators of inflammation at the sites where the IC are deposited. Consequently, extensive inflammation at these sites creates a functional and morphological impairment. Plasmacytosis, hypergammaglobulinemia, glomerulonephritis, arteritis, and liver dysfunctions are the most common symptoms of IC diseases. Immune complex disease related conditions are described in many human and animal diseases i.e. measles virus specific antibodies in children with subacute sclerosing panencephalitis (Mehta et.al.,

1977), parvovirus infections of Aleutian minks (Porter et. al., 1980) , chronic lymphocytic choriomeningitis virus (LCMV) infections of mice (Oldstone et. al., 1983). Except for an earlier communication (Kazmierowski et.al.,1982) in which the presence of herpes virus antigen was shown in IC of patients with erythema multiforme, HSV-induced IC disease has not been reported.

Another example of humoral-immune response-induced IP is IgE-mediated IP. IgE antibodies normally are found in cutaneous sites anchored to mast cells. Their reaction with specific antigens leads to the activation of these potent inflammatory cells. As a result, potent vasoactive and pyrogenic proteins are secreted by mast cells, and these proteins are responsible for local and systemic immunopathological reactions. The reports on anti-viral IgE responses have been scarce. In both mouse and rabbit models, anti-herpes virus IgE responses have been described (Ida et. al., 1983). However, no detailed follow up studies appeared in the literature after these initial findings. As will be discussed below, recent studies in schistosomiasis and leishmaniasis seem to indicate that IgE-mediated IP should be considered as T cell mediated (James and Sher, 1990), since preferential activation of the T_{H2} subset of $CD4^+$ T lymphocytes, which are crucial in IgE synthesis, appears to be the underlying mechanism of IP in many parasite infections.

2.2. T lymphocytes in immunopathological conditions

In T cell-mediated immunopathological conditions, the mechanisms of pathology largely depend on the mode of action of the T cell subset involved. Also, the antigen recognized by these cells and the tissue distribution of the antigen are critical determinants of this process. If the T cell subset involved is a cytolytic one (T_{CTL}), the result will probably be the direct lysis of target cells carrying the immunopathological epitope. The best example for this type of process seems to be the central nervous system (CNS) IP observed in LCMV infections of mice (Allan et. al., 1987). If, however, IP-mediating cells are comprised of DTH-mediating T cells (T_{DTH}), then, inflammation and IP will probably be the result of activated macrophages and other DTH effector cells driven by these T_{DTH} (Clatch et. al., 1986).

Original definitions of T_{CTL} and T_{DTH} were based mainly upon the cell surface expression of CD8 and CD4 molecules, respectively. However, it is becoming increasingly obvious that functional differences between these two subsets are not as great as once anticipated (Braakman et.al., 1987). Thus, one particular T cell subset can exhibit several different activities such as the elaboration of DTH, cytotoxicity, and help for B lymphocytes (Chang et. al., 1990 a, 1990 b, and Yasukawa et. al., 1988). In light of these reports, it would be misleading to conclude that only one immunological mechanism is responsible from an immunopathological disease since one particular cell subset seems to have a role in the development of that particular condition.

As more inquisitive studies are conducted on the functions of T cell subsets, it has become increasingly difficult to ascribe a specific function to a particular T-cell subset (Bottomly, 1988). Nevertheless, in the next section, viral immunopathological conditions will be summarized according to the surface phenotype of immunopathogenic T cells involved.

2.2.1. $CD8^+$ T cell-mediated immunopathology. The two classical functions of $CD8^+$ T cells is cytotoxicity manifested in an MHC-I restricted mode, and suppression of immune responses. Additionally, at least some, $CD8^+$ T cells can provide help in the form of autocrine IL-2 secretion (Mingari et. al., 1984, and Patel et. al., 1988). Thus, one can visualize that all three mechanisms, cytotoxicity, suppression, and helper activity, could be operative in $CD8^+$ cell-mediated pathology. As discussed in detail in the second part of the review, the presence of a $CD8^+$ T cell-mediated IP has been proposed in HSK. Thus, in the next section, $CD8^+$ T cell mediated immunopathological conditions in other viral systems will be outlined.

One of the most scrutinized virus models from the stand point of $CD8^+$ T cell-induced IP is murine LCMV infection. The classical experiments on IP and the adoptive transfer of immune T lymphocytes have been reported by Gilden et. al.(1972a and 1972b) in this model. Intracranial infection of immunocompetent mice with LCMV results in the

fatal inflammation of the choroid plexus and leptomeninges (Allan et. al., 1987).In immunocomprimised animals, a persistent infection is observed, characterized by viral dissemination to all major visceral organs (Buchmeier et.al., 1980). In the acute infection, the dissemination of the inflammatory process in the CNS is closely related to the areas of infection. The presence of viral antigens in the inflammatory sites have been demonstrated by immunofluorescence studies. However, the virus is not directly responsible for the pathology (Allan et. al., 1987). Immune cells responsible for CNS IP have been identified as $CD8^+$ T lymphocytes specific for LCMV antigens, since either cloned ($1\text{-}3x10^6$ cell/mouse, intracranial transfer) or polyclonal $CD8^+$ T ($1.5x10^7$ /mouse) cells could induce IP upon transfer to infected-immunosuppressed syngeneic mice (Beazinger et. al., 1986, and Dixon et. al., 1987). In persistent infections, adoptive transfer of LCMV-specific CTL ($1x10^7$ CTL/mouse, intravenous transfer) also induce a fatal disease, but in this model, the inflammation is mostly confined to the lungs (Byrne and Oldstone, 1986). The specifity of $CD8^+$ CTL in LCMV infection, and T cell receptor (TCR) gene usage by these cells are reported (Yanagi et. al., 1990, Pircher et . al., 1987, and Klavinskis et. al., 1989, Whitton et. al., 1989). Studying the CNS inflammation, Doherty and colleagues (1990) have drawn attention to the virtual absence of $CD4^+$ T lymphocytes in the inflammatory reactions. Since $CD8^+$ cells, in LCMV, are also functional in DTH reactions as well as in class I-restricted cytotoxicity (Ahmed et. al., 1988, Moskophidis et. al., 1990), it is likely that both mechanisms have roles in meningitis.

Hepatitis B virus (HBV) infections are another example of $CD8^+$ T lymphocyte-mediated IP. Hepatitis B virus is assumed not to be cytotoxic to liver cells (Hollinger et. al., 1990). Thus, most of the damage during acute hepatitis may be due to the immune response directed against the virus. Intrahepatic $CD8^+$ T cells from biopsy specimens of patients and the presence of HBV capsid antigen-specific CTL in the circulation lend support to this notion (Mondelli et. al., 1990). To confirm such findings in an *in vivo* setting has been difficult since data gathered from animal studies remains scarce for HBV infections. Recently however, transgenic mice were used to probe the mechanism(s) of IP

in HBV infections. In an elegant series of experiments Moriyama et. al., (1990) proved that indeed HBV envelope protein (env) specific $CD8^+$ lymphocytes are the main cause of hepatic cell injury. In these experiments, expression of the HBV envelope protein was observed in the hepatocytes of transgenic mice. Intraperitoneal adoptive transfer of $CD8^+$ T cells ($1x10^7$ cell/mouse) specific for HBV env (aa 20-41) into syngeneic transgenic mice was able to induce hepatocyte lysis as assessed by histopathological and liver function assays.Thus, these results not only identified the immunopathological T cell subset but also the region of the envelope protein which serves as the immunopathogenic epitope.

An additional viral model of IP is coxsackie virus infections as studied in mice. In this model, immunopathological manifestations are related to myocarditis (Woodruff and Woodruff, 1974). The presence of IP is experimentally supported by immunosuppression - adoptive transfer approaches. After immunosuppression (thymectomy+irradiation+bone marrow transfer), viral challenge does not induce myocarditis in DBA mice. However, $1x10^6$ immune-syngeneic $CD4^+$ T lymphocyte did increase the incidence and the severity of the disease upon adoptive transfer (Blay et. al., 1989). The cell subset involved in the IP varies in different mice strains. In DBA/2 mice, $CD4^+$ cells are implicated exclusively in the IP, whereas in Balb/C mice both subsets ($CD4^+$ and $CD8^+$) are believed to be capable of causing the disease (Van Houten and Huber, 1974). The presence of virus and cardiocyte-specific CTL has been shown (Estrin and Huber, 1974 and Gutrie et. al., 1984). There are also reports indicating that cardiac muscle-reactive IgGs can be detected in the sera of infected mice (Huber and Lodge, 1984). Thus, coxsackie infection of mice is currently considered to be a model system for both antibody and T cell mediated IP.

2.2.2. $CD4^+$ T cell -mediated immunopathology. $CD4^+$ T cells are comprised of functionally different sub-populations (T_{H1} and T_{H2}). $CD4^+$ T cells are involved in IP either by mediating the pathology themselves or providing help to other immunopathological elements. Although $CD4^+$ T cells are implicated in several viral-induced immunopathological conditions, the most definitive and detailed reports on

$CD4^+$ T cell-mediated IP are found in parasite models.

In this section, examples from both viral and parasitic models will be summarized. Since some of the IP mediated by $CD4^+$ T cells manifests itself as an autoimmune type reaction, possible mechanisms of auto-immunity will also be listed. Additionally, several other points on $CD4^+$ T cell-mediated IP such as the subdivision of $CD4^+$ T cells, the effects of the route by which antigen is introduced into organisms, and the role of lymphokines will be discussed. These points are important for a better conceptualization of $CD4^+$ T cell-mediated IP, particularly as it relates to our current model of stromal disease and for future investigations on HSK IP as well.

In several viral infections which are either naturally occurring such as Marek's disease of chickens (Sell, 1987) or model diseases such as Theiler's Murine Encephalitis Virus (TMEV) infections of mice, an autoimmune type of demyelination occurs (Lipton, 1975).In TMEV infection, an animal model for Multiple Sclerosis (MS), early depletion of $CD4^+$ cells causes extensive viral replication with subsequent fatal outcome. If, however, anti-CD4 monoclonal antibody administration is delayed for several weeks, reduced demyelination is observed and the clinical symptoms are ameliorated (Welsh et. al., 1987). TMEV-specific DTH, antibody, and lymphoproliferative responses have been shown in affected mice (Clatch et. al., 1986). Demyelination in these cases is believed to be the result of DTH type reactions mounted mainly by $CD4^+$ T cells (Clatch et. al., 1986). However, contradictory findings on the identity of immunopathogenic cells exist (Rodriguez and Sriram, 1988). Accordingly, $CD8^+$ T cells appear to play a role in demyelination since the depletion of $CD8^+$ T cells in infected mice reduces the degree of demyelination (Rodriguez and Sriram, 1988). Thus, it is possible that both T cell sub-populations have some role in the disease process. Early reports indicated that the target antigen recognized by immunopathologic responses in TMEV induced diseases is myelin (Clatch, et. al., 1985, and 1986), however, this hypothesis is refuted on the ground that T cells obtained from affected mice do not cause demyelinating diseases in the uninfected syngeneic mice upon transfer (Rodriguez et. al. 1986) . Currently, the antigen recognized by T cells which

induce demyelination is obscure. It is argued that if the antigen is a self molecule, its expression must be associated with TMEV-infection in some way (Rodriguez et. al. 1986).

Another picornavirus model for MS is Encephalomyelocarditis virus-induced CNS disease which resembles the postinfectious demyelination observed after several human diseases such as rubella, measles, and chicken pox. Again in this model T cell depletion ($CD4^+$ and $CD8^+$) reduces IP (Sriram et. al., 1989).

In the field of immunopathogenesis and auto-immunity, one of the major questions the researchers have been facing is how the immune system becomes sensitized to a self protein. There are several hypothesis put forward to explain this phenomenon. One of the more favored hypotheses suggests that immune surveillance in immunologically privileged sites such as CNS, cornea, and testis, normally is less than optimal and consequently antigenic moieties in these sites are hidden from immune system's watchful eye (Streilein and Wegmann. 1987) After the infection however, the lymphatic drainage in these sites is increased and self epitopes are introduced to the immune system. A less favored hypothesis predicts that self-protein cross-reactive epitopes on the invading virus induce an immune response. According to this molecular mimicry hypothesis, the myelin is victimized as a result of this unlucky resemblance (Fujinami, 1989, and Oldstone, 1989). Both hypotheses, however, agree on the fact that the incomplete elimination of self-reactive T cells during ontogeny creates a potentially dangerous situation and on occasions autoimmune reactions may be triggered (Sinha et. al., 1990). It has also been argued that viral infections can induce the over-expression of MHC antigens in tissues which normally express very little or no histocompatibility molecules (Massa et.al., 1986). Thus, the presentation of self antigens might be facilitated as a result of virus infections. Another hypothesis for the induction of autoimmune responses following virus infections proposes that viral elements can alter the regulatory mechanisms of immune cells such that a polyclonal immune response is raised against self proteins and many other proteins as well (Rickinson et. al., 1985).

Another virally induced immunopathological condition where the pathology is thought to be mediated by $CD4^+$ T cells is Borna disease virus infections. Borna disease

virus, a relatively uncharacterized virus causes an endemic progressive encephalomyelitis in sheep and horse. A rat model for the disease has been established. As little as $1x10^6$ Borna virus specific $CD4^+$ T cells are sufficient to induce encephalomyelitis in immunosuppressed rats upon adoptive transfer, whereas in negative control rats which did not receive cells, no apparent disease developed (Richt et. al., 1989). Histopathological findings in recipient mice were similar to immunocompetent positive control animals where the inflammation is confined to the gray matter (Richt et. al., 1989).

One of the most studied $CD4^+$ lymphocyte-induced IP model is murine leishmaniasis (Hill et.al., 1989, Liew et. al., 1990, Moll and Rollinghoff, 1990, and Muller et. al., 1989). Initially, depending upon the mice strain selected, immune $CD4^+$ T lymphocytes were reported to be either immunopathogenic or protective after challenge infection. For example, adoptive transfer of immune, syngeneic $CD4^+$ cells to naive Balb/c mice exacerbated the disease, and resulted in death (Liew 1987). However, in C57BL/6 mice, such transfer did not result in IP (Liew et.al., 1985). Later studies have shown that both protective and disease promoting cells exist in CD4+ T lymphocytes of a given mouse strain. Accordingly, intraperitoneal adoptive transfer of $1x10^6$ CD45-low (-) subpopulation of $CD4^+$ T cells could increase the severity of the disease in nude Balb/c mice, whereas CD45-high (+) $CD4^+$ cells could induce protection in the same adoptive transfer model (Moll and Scollay, 1989).

Further analysis of $CD4^+$ T lymphocytes in murine leishmaniasis uncovered a fundamental difference in the lymphokine secretion pattern of $CD4^+$ T lymphocytes (Heinzel et. al., 1989). These reports have provided strong experimental evidence to support the elusive concept of two functionally different $CD4^+$ T cell subsets, namely T_{H1} and T_{H2} cells (Mossmann and Coffman, 1989). The role of differentially secreted lymphokines in the disease process was further established by anti-lymphokine antibody experiments. Anti-lymphokine antibody experiments provided evidence that gamma interferon secreted exclusively by T_{H1} has a central role in resistance and it is the

predominantly detected lymphokine in the lymph node cells of mice recovering from the infection. In contrast, IL-4 produced mainly by T_{H2} is the abundant lymphokine in susceptible mice and very little or no detectable gamma interferon has been found (Belosevic et. al., 1989, and Heinzel et. al., 1989). However, in another study (Sadick et. al., 1990) the role of gamma-interferon in the protection from leishmaniasis was discounted because mice that had been given anti-gamma-IFN were adequately protected if they were also administered anti-IL-4 antibodies at the same time.

The reports on different subsets of $CD4^+$ T cells have, in a sense, revolutionized the old concept of suppression of $CD4^+$ T cells by $CD8^+$ suppressor cells. It may not be surprising to observe in other experimental models that preferential activation of one T cell sub-subset and subsequent over-expression of lymphokine(s) is responsible for the outcome. By doing so, one particular T cell subset steals the show rather than actively suppressing the other arm of the immune system.

Another interesting point which should be made on the subject of T_{H1} and T_{H2} cells is that a recently discovered lymphokine, IL-10, might exert a regulatory role on the functions of these cells. Accordingly, IL-10 is produced by T_{H2} cells and can down-regulate the secretory functions of T_{H1} cells (Fiorentino et. al., 1989). Thus, for all practical purposes, it acts like the long searched for suppressor molecule.These properties of IL-10 make it a perfect candidate lymphokine to play a central role in the IP observed in leishmaniasis. However, this point must still be proven *in vivo*. One obvious approach would be to utilize antibodies against IL-10 to neutralize its function. There have been many reports attributing suppressive functions to a variety of cytokines such as gamma interferon's effect on B lymphocyte function (Mossmann and Coffman, 1989) or tumor growth factor-beta's suppressive activities on T lymphocytes (Fontana et. al., 1989). However, to translate such *in vitro* findings into concrete *in vivo* results seems to be an impossible task, because many of the cytokines are produced by more than one cell type and usually exhibit pleiotropic effects on different cells. Thus, to study the exact activities of a cytokine in a given microenvironment and to reach a credible conclusion has been frustrating at many times.

Reports on the presence of different T cell subsets recognizing the same pathogen imply that the infectious organisms possess functionally different antigenic structures. Some of these epitopes stimulate a protective immune response. In contrast, the recognition of putative immunopathological and/or suppressive epitopes will negate the function of protective immune response. One such immunopathological epitope has been recently identified in the *L. major* model. An octomeric amino acid sequence from the p183 protein of *L. major* seems to preferentially induce the T_{H2} subset in Balb/c mice (Liew et. al., 1990). This sequence is a repetitive motif in the protein and priming mice with the peptide corresponding to this sequence induces a proliferative response in lymph node cells exposed to soluble extracts of *L. major*. In response to *L. major*, lymph node cells secrete abundant IL-4 but very little IL-2 and gamma interferon. Immunization with p183 , instead of leading to resistance against the disease, worsens the clinical symptoms monitored during subsequent *L. major* challenge (Liew et. al., 1990).

The presence of different epitopes on pathogens indicates that the antigen presenting machinery of the host may play crucial role in the outcome of the immune response. It has been long known that the route by which the antigen is introduced into the host and the delivery vehicle used are important in determining which type of antigen presenting cells (APC) will handle the antigen.Thus, several groups have studied the effects of different immunization routes on the immunity induced against leishmaniasis. The consensus reached from these studies is that intravenous (iv) introduction of the antigen preparation induced a protective immunity against the protozoa, whereas subcutaneous (sc) immunization with the same preparation induced no such effect (Liew et. al., 1985). Moreover, adoptive transfer of $1x10^7$ or more splenic T lymphocytes obtained from mice immunized *via* the iv route (Ti) could provide protection from *L. major* challenge in irradiated recipients, whereas the same number of T cells from mice immunized *via* sc route (Tsc) increased the disease in recipient mice (Liew et. al., 1987). Additionally, the protective effects of Ti could be negated by the injection of a higher number ($1x10^7$ or $5x10^7$ cell/mouse) of Tsc into the recipients at the same time (Liew et. el., 1987). Thus, it is argued that systemic immunization preferentially induces T_{H1} cells whereas subcutaneous

priming activates T_{H2} cells (Scott, 1990).

Each virally induced IP represents a unique challenge to the investigators in the field. However, as surveyed in the previous section, many of the approaches used during the studies have some common aspects. Consequently, a method proven to be useful in one model is usually adapted to others. With this perspective in mind, in the next section, HSV-induced immunopathological conditions and more specifically HSK will be discussed.

3. IMMUNOPATHOLOGY OF HSV INFECTIONS

Herpes simplex virus infections in humans are mostly confined to mucocutaneuos tissues and the lesions are believed to be the result of the cytopathic effect of the virus (Whitley, 1990). Virus is either acquired from exogenous sources or mobilized from latent sites before reaching susceptible cells. Infected cells upon lysis release progeny virus which can then infect neighboring cells (Whitley, 1990). This cycle continues until the immune system is able to intervene and eventually halt this process. Thus, the tissue damage induced during most HSV infections is caused by the virus rather than the immune response. Thus, the role of IP in HSV infections is usually overlooked, and as discussed below, apart from herpetic stromal keratitis, and to a degree uveitis, herpes simplex infections of humans are not considered in the general topic of IP.

One of the most common immunopathological processes encountered during many viral infections is an exanthema observed around the initial infection site (Sell, 1987). Exanthema formation is most likely the result of a DTH reaction. (Sell, 1987). Although the redness in the base of the herpetic lesions might be the result of such a reaction, the duration and the severity of the these lesions are inconsequential. Thus, no detailed study has been conducted on herpetic exanthemas. More serious and consequential immunopathological conditions develop if the regenerative capacity of cells destroyed during the immunopathological process is limited. Additionally, if functions of the tissues are permanently impaired as a consequence of the inflammatory or reparative process, IP could be the single most damaging result of the virus infection. One such condition in human herpetic infections is believed to be represented by corneal stromal infection (Rouse, 1985). Corneal infection starts as the infection of superficial cornea. Primary lesions are confined to the epithelium. In the recurrent episodes of the disease however, deep stromal layers are involved and as a result of complex virus-immune system interactions, loss of stromal tissues leads to scar tissue formation, permanent opacity of the cornea, and blindness (Daniels, 1982, and Vaughan, 1989). As discussed below, data gathered from animal models suggest that this process might be the result of IP triggered by recurrent HSV infections of the cornea (Kaufman, 1978, Rouse, 1985).

Another immunopathological condition in HSV infections is uveitis. Uveal tract infections of humans by HSV is relatively uncommon and data gathered from these cases are scarce. Uveitis in animal models is induced by the intravitreal introduction of HSV antigens into rabbits recovering from primary infections (Oh et. al., 1985). Flare in the uveal tissues and inflammatory cells collected in the anterior chamber (AC) are considered as signs of uveitis. Results obtained from this model suggest that secondary uveitis might represent an immunopathological condition. Secondary uveitis develops independently of the replicative capacity of the injected HSV (i.e. UV-inactivated HSV can induce the disease as well as live virus). Also, efforts to recover the virus from infected uveal tissues have been unsuccessful implying a minimal role for virus-induced damage (Oh, 1976). The mechanism of IP and the immune elements involved have not been studied in the HSV uveitis model.

In another immunopathological uveitis model, rat experimental autoimmune uveitis (EAU), immunization of animals with S antigen in Freund's Complete Adjuvant induces autoimmune reactions and the pathology is mediated by $CD4^+$ T lymphocytes (Mochizuki et. al., 1985). A well studied adoptive transfer model exists for EAU (Mochizuki et. al., 1985, Caspi et. al., 1986, Palestine et. al., 1986,). Accordingly, as little as $5x10^6$ *in vitro* S-antigen-stimulated lymph node T lymphocytes obtained from S-antigen immunized rats could induce the disease in recipient rat (Caspi et. al., 1986). Following intraperitoneal adoptive transfer, most T lymphocytes leave the peritoneal cavity in the first 24 hr, and end up in the liver, spleen and thymus (Palestine et. al., 1986). Some of the uveitogenic T lymphocytes, however, migrate to the uveal tissues, and initiate the inflammation (Palestine et. al., 1986). Anti-IA antibodies administered before the immunization did indeed inhibit the development of uveitis indicating a role for Class-II restricted cells in the immunopathologic process (Rao et. al., 1989). Recently it was also reported that a majority of uveitogenic T cells express T cell receptor molecules belonging to the V-beta 510 and V-alpha 510 families (Gregorsan et. al., 1991). Similar studies remain to be conducted in the HSV uveitis model.

3.1. Herpes Simplex Virus-Induced Keratitis (HSK)

Ocular herpes infections of humans are usually caused by HSV-1 (Hyndiuk and Glasser, 1986). In primary ocular HSV infections the conjunctiva and epithelium of the cornea are the most common sites of infection and in immunocompetent patients recovery without any serious consequence is observed (Daniels, 1982). However, a significant number of patients with primary infections experience recurrences (Kaufman, 1978). Primary ocular infections are observed in patients with a history of HSV infections although it is also likely that ocular tissues might be the initial HSV infection sites. Ocular pain, loss of corneal sensitivity, photophobia, and tearing are non-specific initial symptoms experienced during primary attacks (Daniels, 1982 and Vaughan, 1989). The most common form of epithelial keratitis are dendritic ulcers characterized the branching lesions which cause superficial opacities. During the recurrent episodes the corneal stroma is usually involved to some extent and depending upon the degree of stromal involvement, the prognosis of the disease can take a serious path (Kaufman, 1978). In stromal inflammation, the characteristic feature is disciform keratitis which develops as a result of varying degrees of edema and the collection of inflammatory cells in stomal tissues. Both forms of keratitis are usually self limiting and with the proper management, an adequate healing can be obtained (Daniels, 1982). However, in some cases (25% of total primary infections) recurrences are observed. Topical anti-virals are effective in the management of the primary HSV keratitis. However, corticosteroid therapy, although able to provide temporary comfort for patients experiencing the epithelial keratitis, is contra-indicated.This is because corticosteroid therapy can cause immunosuppression, increased collegenase activity, increased viral replication, and a heightened susceptibility to secondary infections (Oh, 1970, Robbins and Galin, 1975, and Vaughan, 1989). In stromal keratitis, however, corticosteroid therapy is advised since immune factors as well as the virus itself is implicated in the pathogenesis. Reports in the literature also indicate that antiviral therapy has no beneficiary effects on the prognosis of stromal disease (O'Brien, 1986). As a matter of fact initial studies designed to elucidate the role of the immune response in HSK were instigated from these clinical observations as well as from reports showing that the virus is very rarely isolated from stromal lesions.

3.1.1. Animal models of herpetic stromal keratitis. Two of the most prominent animal models of HSV-induced corneal infections are the rabbit and the mouse model (Metcalf et. al., 1976, O'Brien, 1984, Opremcak et. al., 1990, and Stulting et. al., 1985). In both models, although it is not absolutely required, the corneal epithelium is scratched and infectious virus is inoculated onto the cornea (Myers-Elliot et. al., 1983). During the next several weeks, initially an epithelial and then stromal keratitis is observed (O'Brien, 1984). In rabbits the disease usually results in a necrotizing keratitis if a large enough inoculum is used (Metcalf et. al., 1976. In some strains of mice, such as C57BL/6 and C3H, the animals are resistant to herpetic stromal keratitis. In other strains such as Balb/c and A/J mice, a characteristic HSK develops over the next several weeks (Opremcak et. al., 1990). Depending upon the size of the inoculum the virus can establish a latent infection in both animal models without causing any initial disease. In mice, recurrences do not occur spontaneously. Even experimental manipulations are ineffective in mimicking the reactivation as it occurs in humans (O'Brien, 1984). Rabbits, however, seem to be a good animal model for such latency-reactivation studies since virus can be recovered from ocular surfaces after such manipulations as iontophoresis, and surgical stimulation (Nesburn et. al., 1977, and Se Kwon et. al., 1981).

The rabbit model of HSK is also preferred in experiments assessing the effects of antivirals since the disease is easy to produce and to monitor (O'Brien, 1984). However, rabbits are known to be more susceptible to herpetic infections than humans. Thus, the data obtained from these animals should be evaluated with caution. Furthermore, for studies designed to understand the role of individual immune factors in HSK, rabbits do not offer a good model since the immune system of rabbits has not been as thoroughly studied as is the case for mice. Additionally, reagents available for immune function studies in the rabbit are limited compared to availability of mouse reagents. Apart from these considerations, HSK induced in rabbits and mice display a similar histopathology (Metcalf and Reichert, 1979, and Opremcak et. al., 1990). In both models, the initial histopathological finding is a PMN infiltrate spread over the corneal epithelium 3-7 days following HSV challenge. During this period, lymphocytes start to infiltrate the limbus. The limbus may be the first

site where lymphocytes meet antigen. Thus, the limbus may act as lymphoid organ (Metcalf and Helmsen, 1977). During the second week of infection, the major findings are neovascularization of the corneal stroma, loss of epithelial integrity, and the collection of PMN, lymphocytes, and macrophages in and around the capillaries. During this period, lymphocytes are found in close contact with keratinocytes undergoing degenerative changes. This might be indicative of a T-cell-mediated attack on infected keratinocytes (Metcalf and Helmsen, 1977, and Metcalf and Kaufman, 1976). Later in the disease process, the degeneration of corneal layers increases and results in the loss of keratinocytes leaving permanent scarring. Scar tissue formation is believed to be the most damaging result of HSK not only in animals but also in humans (Hyndiuk and Glasser, 1986).

3. 1. 2. Herpetic stromal keratitis as an example of immunopathology. Initial experimental support for the presence of an immunopathological corneal destruction during HSK comes from studies with nude mice (Metcalf et. al., 1979, and Russell et. al., 1984) . Attempts to induce HSK in nude mice have failed in the sense that permanent opacity of cornea and loss of vision are not observed in these mice. Furthermore, nude mice do succumb to encephalomyelitis, stromal disease with long lasting symptoms is not experienced. During the first several days of infection, a superficial inflammation is observed which subsequently clears (Russell et. al., 1984). Control animals, (euthymic Balb/c mice), experience HSK with characteristic symptoms (i. e. edema collection in stromal layers, and neovascularization of cornea followed by inflammatory cell influx into the central cornea. Further support for IP is provided by adoptive transfer experiments in which nude Balb/c mice were transferred with HSV immune cells from euthymic mice (Metcalf, 1984, and Russell et. al., 1984). After intraperitoneal or subcutaneous immunization of normal mice, unfractionated splenocytes are transferred to nude mice which had been previously challenged on the cornea with virus. This treatment was able to induce HSK in nude mice with comparable severity to normal animals. In these experiments, the authors observed that the SC route of immunization of donor mice was more effective in inducing the disease in recipient mice. Moreover, splenic adherent cells seem to potentiate the immune splenocytes' ability to induce HSK in nude mice, since after

elimination of adherent cells, the remaining cells do not induce the disease (Metcalf, 1984). The hypothesis put forward is that essentially two population of immune cells exist in donor splenocytes (i. e. protective versus disease-inducing populations). The latter cells are presumed to be in the adherent population (Metcalf, 1984).

3.1.3. T cells in the immunopathogy of herpetic stromal keratitis. More recent studies have concentrated on the identification of those immune T cells responsible for IP (Hendricks et. al.,1989a and 1989b, and Newell et. al., 1989a and 1989b). There are two competing schools of thought on the identity of the T cell subset involved in HSK IP. As discussed below in detail, findings reported mainly from our laboratory support the view that $CD4^+$ T cells are the primary immunopathogenic cells potentiating pathology in the corneal stromal tissues. The second hypothesis fostered by Hendricks and colleagues favors the idea that HSK IP is the result of damage induced by anti-HSV CTL responses mediated mainly by $CD8^+$ T cells.

By using monoclonal antibodies specific for T cell subsets in a neagtive depletion approach, Newell et al., (1989a and 1989b) studied the role of T cells in HSK. In this model, T cell subsets (i.e. $CD4^+$, and $CD8^+$ cells) of Balb/c mice were eliminated from the circulation by the repeated administration of mAbs. More than 95% of the circulating target T cell populations were eliminated by this treatment as determined by Fluorescence Cell Sorter Analysis (Newell et. al., 1989 a). These mice which lacked either T cell subset were then later challenged on the cornea with the RE-strain of HSV. The main finding was that the depletion of $CD4^+$ T cells in normal Balb/c mice significantly reduced the clinical symptoms of HSK (Newell et. al., 1989 a and 1989 b). Interestingly, after the elimination of $CD8^+$ T cells, mice experienced more severe stromal disease and the symptoms of encephalomyelitis.

These results which implicate a $CD4^+$ T cell-mediated IP in HSK induced by the RE strain of HSV have been confirmed by others (Hendricks and Tumpey, 1990). Interestingly enough though, they found that IP could also be mediated by $CD8^+$ T cells if the KOS

strain of HSV is used as the challenge virus. These results were obtained from *in vivo* T cell depletion experiments. Currently, there is no adequate explanation as to why two different sub-populations of T lymphocytes are activated in a almost mutually exclusive fashion by different virus strains. Furthermore, it is not known whether these HSV strains differ antigenically.

In earlier reports (Ksander and Hendricks, 1987, and Hendricks et. al., 1989), the same researchers used a novel but rather indirect approach to show the role of MHC-Class I-restricted CTL in HSK. The introduction of HSV into the anterior chamber (AC) of mice induces a state of immune tolerance characterized by the lack of CTL and DTH responses. Anti-viral antibody responses, however, remain intact. A simultanous AC+topical corneal HSV challenge do not cause HSK implicating either CTL or DTH response is mediating HSK. Because when both responses are tolerized no disease could develop (Ksander et. al., 1987). When an HSV-mutant lacking glycoprotein C was used to inoculate the AC, mice could still raise a normal DTH response whereas Class I-restricted CTL responses were impaired (Hendricks et. al. 1989b). In the case where the AC injection was done with the mutant virus and corneal infection with the wild type HSV, mice still were unable to develop HSK implicating a Class I-restricted $CD8^+$ T cell mediated IP, because in these mice only Class-I-restricted CTL responses were lacking (Hendricks et. al., 1989). Although the results have not been confirmed by others, the argument presented seems persuasive.

One additional point which needs to be addressed on this issue is the nature of the split tolerance caused by the AC immunizations. The original reports on the immune tolerance following AC priming indicated that the only impaired immune function in these cases is the DTH response. Furthermore, an adequate CTL response had been detected against antigens introduced *via* AC route. (Whittum et. al. 1983, Streilein, 1987, and Niederkorn, 1990).Thus, there is a significant discrepancy in the nature of the split tolerance reported by Hendrick's et. al. (1989 a and 1989 b) and those of the others. These differences remain to be addressed in a comprehensive study.

After identifying the T cell subset responsible for the development of a particular

immunopathological condition, the next step is to study how the responsible T cells induce the destruction and what mechanisms are utilized during the course of the disease. Since a particular T cell subset can utilize several different effector mechanisms, the studies needed to answer these questions are difficult to design and the answers are not clear cut (Doherty et. al., 1990). Added uncertainties in the case of HSK come from the fact that assays measuring the functions of T cells in HSV mice models usually require *in vitro* stimulation steps increasing the possibility of introducing artifactual elements to data. Furthermore, the microanatomy of corneal tissues makes *in situ* analysis of T cell function almost impossible (Streilein, 1987). Thus, T cells obtained from local lymph nodes (retropharyngeal and cervical lymph nodes) seem to be the best candidates for study. Since *in vivo* depletion experiments have indicated that $CD4^+$ T cells are crucially involved in HSK IP, further efforts should be concentrated on the defining the function of these cells.

One of the more universally accepted functions of $CD4^+$ T cells is their participation in DTH reactions. This notion has also been proven in the murine HSV model (Nash et. al., 1981, and Rouse, 1985). Furthermore, Newell et. al., (1989 a) indicated that in both normal and $CD8^+$ cell-depleted mice experiencing HSK, a significant DTH response was mounted against HSV antigens. Furthermore, histopathological examination of diseased corneas showed that the collection of inflammatory cells in the corneal tissues is reminiscent of conventional DTH reactions. Another approach to assess the role of DTH in immune responses is to determine the lymphokine secretion pattern of $CD4^+$ cells. Gamma-IFN and IL-2 are predominantly secreted by $CD4^+$ T cells mediating DTH reactions (Mossmann and Coffman, 1989). In this context, the roles of gamma interferon and IL-2 in HSK might be important, if indeed DTH responses are the primary mechanism of IP in HSK.

Another mechanism by which $CD4^+$ cells might be functional in HSK IP is direct cytotoxicity against virus-infected corneal cells. We recently described the presence of $CD4^+$, Class II restricted CTL in the HSV murine model (Kolaitis et. al., 1990). In this system, it was shown that $CD4^+$ T cells could lyse HSV-infected syngeneic, IA^+ targets

effectively after an initial priming via footpad and ear pinnae routes. Thus, it seems that a Class-II restricted CTL response is an integral part of the anti-herpetic immune response in Balb/c mice. It is possible that $CD4^+$ CTLs are also present in the retropharyngeal and cervical lymph nodes of mice with HSK. Their migration to the inflamed cornea and their cytotoxic activities on infected corneal cells which display HSV antigens along with the IA molecule might be another possible mechanism of IP. The contribution of $CD4^+$ CTL in the overall development of HSK IP is difficult to assess. However, the presence of MHC-Class II-expressing cells in the limbus, central and peripheral cornea have been shown in man and mice with HSK (McBride et. al., 1988, and Opremcak et. al., 1990). Furthermore, it is becoming more evident from human studies that $CD4^+$ CTL may play a significant role in defense against HSV because $CD4^+$ CTL are readily detectable at high frequencies (Schmid, 1988,and Yasukawa et. al.1988). As shown in immunohistochemical stainings, infected keratinocytes express MHC-Class-II antigens during the first day of infection (Cunningham et. al. 1985). Moreover, keratinocytes, after treatment with gamma-IFN, could present HSV antigens to $CD4^+$ CTL in chromium release assays (Cunningham et. al.1989). Thus, it currently appears that $CD4^+$ CTL are an important part of overall anti-herpetic immune response in both humans and mice. Consequently, they might be involved in the development of HSK.

The question of how $CD4^+$ CTL display their cytotoxic activities has not been studied in detail. It has been shown in the influenza system that Class II-restricted CTL secrete tumor necrosis factor (TNF) and the lysis of target cells is accomplished primarily via TNF (Tite, 1990). A similar mechanism might be operative in HSV although no data exist to support this hypothesis.

3.1.4. The role of non-T cell-mediated mechanisms in herpetic stromal keratitis. The effects of B cells, and antibody-mediated IP have not been addressed adequately in HSK. In light of the recent findings which indicate a role for CD4+ T cells in the disease process, the search for immunopathological antibodies should be more

intensively pursued. It is entirely possible that CD4+ cells might also be involved in antibody production by providing help to B cells. Immunohistochemical data proving the presence of plasma cells in the limbus and in corneal tissues further support this hypothesis (Metcalf, 1976 and Meyers-Elliott and Chitjian, 1981).One direct approach to answering the question of the role of B cells in HSK IP was reported by Jordan and colleagues (1983). They indicated that elimination of B cells reduced the clinical symptoms of HSK in mice; however in that study, experiments were terminated at only 4 days post infection. Thus, the findings related to the early clinical symptoms may not reflect how such treatment will affect the stromal disease which is at its peak during the second week of infection.

Another host defensive element which may be involved in HSK are polymorphonuclear leucocytes (PMN). The possibility of a primarily PMN mediated corneal destruction during HSK has been debated previously (Meyers-Elliott and Chitjian, 1981). Polyclonal anti-PMN antibody treatment of rabbits reduced the severity of the disease significantly thereby implicating an immunopathological role for PMN. In addition, histopathological examination of diseased corneas shows large number of PMN during all stages of HSK (Metcalf and Reichert, 1979, Meyers-Elliott and Chitjian, 1981). However, the impact of anti-PMN antibody treatment on lymphocyte subsets, particularly on T cell subsets was not determined (Meyers-Elliott and Chitjian, 1981). A significant reduction in neutralizing antibody titers against HSV was observed, implicating a defect in helper T cells or B cells. Thus, it is possible that polyclonal anti-PMN antibody treatment, along with the depletion of PMN, had indeed eliminated some of the lymphocytes whose functions are crucial in the disease process. Nevertheless, it would be unwise to discount the role of PMN in HSK IP entirely. A well defined antibody against PMN could be used to determine the precise role of PMN. It is theoretically possible that $CD4^+$ T cells, B cells, and PMN are all involved in the development of HSK since all are functional in antibody production and antibody mediated-virus clearance. The process of $CD4^+$ T cell priming in ocular tissues, local anti-HSV antibody production, and the elimination of antibody-HSV complexes from corneal tissues by PMN could induce the IP collectively.

An additional immune system-related factor which is thought to influence the

development of HSK is the isotype of immunoglobulin heavy chain gene used by the host. The effects of IgH chain gene products on immune responsiveness and/or immune suppression have been hypothesized previously in other systems (Sy and Benacerraf, 1988). However, our understanding of the phenomenon is far from satisfactory. So far, it has been demonstrated that some suppressive factors are restricted in their action to a particular IgH chain gene isotype similar to those imposed by MHC molecules, (i.e. suppressor factors can only suppress the function of lymphocytes in mice which are syngeneic at the IgH locus). Furthermore, the IgH locus of the host seems to have an influence on both the T cell responses and the spectrum of the antibody response to a particular antigen (Dorf and Benacerraf, 1984). In the HSK system, Foster and colleagues (1989) defined different disease patterns in IgH chain congenic mice strains. Accordingly, in mice whose genetic differences are confined to the IgH chain gene only, the susceptibility to HSK differs significantly implying that the IgH phenotype of the animal plays a role in HSK IP. However, at present how this role is played remains to be elucidated

4. RATIONALE FOR THE PRESENT STUDY

As indicated in the previous sections of this review, a logical approach for studying immunopathological conditions regardless of their cause has been to establish a reliable and reproducible animal model. Only then is the stage set where one can scrutinize the immune elements and mechanisms involved in the process. In the case of HSK, a well accepted animal model has been established. Initial studies using the Balb/c mouse model indicate that this model should be useful for detailed future experiments. Furthermore, T cell depletion and nude mice studies support the view that T lymphocytes and particularly $CD4^+$ T cells are the primary mediators of IP in HSK. However, more definitive experimental evidence is needed in order to conclude which T lymphocyte subset is centrally involved in HSK IP. Thus, the role played by T lymphocytes in the disease process needs to be studied in a well controlled experimental setting. For this reason, we planned to study the effects of individual T cell subsets using an adoptive T-cell transfer approach. One advantage provided by this method is that the role of the transferred cell population could be assessed in an environment isolated from other T cell populations since the recipient mice lack T lymphocytes of their own.

Once the individual immunopathogenic elements are elucidated, the next logical step is to study how the disease is produced and what are the mechanisms of IP. Since $CD4^+$ T cells can exhibit multiple functions in immune responses, it is of interest to determine whether $CD4^+$ T cells primed as a consequence of the corneal infection are capable of utilizing different effector functions. Thus, the second part of the studies presented here were designed to probe this aspect of HSK IP. For this purpose, the cytotoxic activities of $CD4^+$ T cells isolated from the local lymph nodes of mice with HSK were determined. The main goal of most of the studies related to immunopathogenic processes was to provide a better understanding of the disease with the hope that researchers will be in a more favorable position to intervene, to modify, and possibly to reverse the process. Thus, experiments planned in this dissertation were aimed at enhancing the understanding of the disease process in HSK.

5. SPECIFIC AIMS

1. To establish an adoptive transfer model of HSK in Balb/c mice. For this purpose:

a. A recipient mouse group which lacks functional T cells was generated.

b. T lymphocytes obtained from donor mice were transferred to the recipient group, and the development of HSK in the recipient mice was monitored. The disease potentiated by adoptively transferred cells was compared to that of normal mice.

2. Cytotoxic activities of $CD4^+$ T lymphocytes isolated from local lymph nodes of mice with HSK were determined. This activity was determined in both normal and $CD8^+$ T cell depleted mice.

3.The presence of MHC-II expressing cells and T lymphocytes in the diseased corneas was investigated using immunohistochemistry.

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PART II:

ADOPTIVE TRANSFER MODEL OF HERPETIC STROMAL KERATITIS (HSK)

1. ABSTRACT

To investigate the role of T cell subsets in the development of HSK in a well defined model, we utilized an adoptive transfer approach in which thymectomized and T-cell depleted mice [T(-)] were reconstituted with different numbers of syngeneic immune T lymphocytes prior to topical corneal challenge with the RE strain of HSV-1. Either *in vitro* stimulated or unstimulated immune T cells obtained from cervical and retropharyngeal lymph nodes (LN) of mice with HSK were used in adoptive transfer experiments.

Although T(-) mice developed an initial epithelial inflammation, stromal keratitis did not occur. Reconstitution experiments revealed that mice which received $2x10^7$ or more unfractionated immune T cells could develop HSK lesions of comparable severity to immunocompetent control mice. In T(-) mice reveiving CD8-depleted T lymphocytes, even less T cells ($1x10^7$/mouse) were able induce significant HSK. In contrast, mice which received similar or increased numbers of CD4-depleted T lymphocytes did not develop HSK. Unfractionated cells or CD8-depleted cells alone transferred without prior *in vitro* stimulation step were also able to potentiate the disease in the recipient mice. Immune T lymphocytes transferred to mice which were mock infected on the cornea did not develop HSK, indicating that the immunopathogenic cells were virus specific and not merely reacting to autoantigens. Histopathological examination of the diseased corneas demonstrated that the stromal inflammation in both euthymic normal, and T(-)-reconstituted mice was characterized by extensive PMN infiltration. Scattered lymphocytes, and occasional macrophages were also observed. These results provide further evidence that HSK represents an immunopathological process mediated mainly by $CD4^+$ T cells. Moreover, the model described here is a useful one to further elucidate the immune factors involved in HSK.

2. INTRODUCTION

Herpes simplex virus (HSV)-induced stromal keratitis (HSK), the leading infectious cause of permanent blindness in Western countries is believed to be the result of complex interactions between the virus and host immune components (McGill and Scott, 1985). Recent polymerase chain reaction and *in situ* hybridization studies have shown that viral DNA is present in the corneal tissues during the latent period of infection (Sabbaga et. al., 1988, Opehshaw et. al., 1990) although efforts to isolate actively replicating virus from the cornea during HSK were proven negative (Dawson et. al., 1968, and Hogan et. al., 1964). The abundance of empty viral capsids and incomplete virions in the corneal stromal cells from patients with recurrent HSK supported the view that corneal pathology could be mediated primarily by non-viral factors (Meyers-Elliott et. al., 1980). Additionally, immunosuppressive therapies have long been known to favorably effect the clinical outcome of HSK (Hyndiuk and Glasser, 1986). Collectively, these observations point to the likelihood that HSK represents an immunopathological reaction, a notion support by numerous observations in a mouse animal model. Accordingly, Balb/c nude mice lacking functional T lymphocytes do not develop characteristic HSK symptoms seen in euthymic mice upon corneal HSV challenge (Metcalf et. al.,1979). Moreover, the adoptive transfer of immune cells obtained from syngeneic mice could potentiate the stromal pathology in the nude mice (Metcalf, 1984, Russell et. al., 1984).However, in the nude mouse model, HSV challenge frequently causes encephalomyelitis (Metcalf, 1984, Russell et. al., 1984) and a spreading dermatitis which makes use of the model system difficult.

More recently, further support for an immunopathological role of T cells in HSK came from experiments in which T cell subsets were depleted by the *in vivo* administration of specific monoclonal antibodies (mAb). By this approach $CD4^+$ lymphocytes appeared to be mediating the pathology with $CD8^+$ cells seemingly playing a protective role (Newell et. al., 1989a, and 1989b). However, some workers have advocated that HSK is primarily mediated by $CD8^+$ lymphocytes participating in a cytotoxic reaction (Hendricks et. al.,

1989). To further understand the role of different T cell populations and their possible interaction, we have in this communication established a T cell deprived model which is suitable for adoptive transfer studies. Our results provide further evidence for an immunopathological role of $CD4^+$ T lymphocytes in potentiating HSK.

3. MATERIALS AND METHODS

3.1. Animals

In all experiments, male Balb/c mice (Harlan Sprague Dawley, Indianapolis, IN) at the age of 5-6 weeks were used.. Animals were housed at the animal facilities of the University of Tennessee, Knoxville, TN. All experimental procedures were in complete agreement with the Association for the Research in Vision and Ophthalmology (ARVO) resolution on use of animals in research.

3.2. Virus

RE- strain of HSV-1 propagated in Vero cells were used throughout the study. Vero cells were grown in McCoy media (GIBCO Laboratories, Grand Island, New York, NY.) supplemented with penicillin (100 U/ml) (GIBCO), streptomycin (100 ug/ml)(GIBCO), and 5% heat inactivated (HI) donor calf serum (GIBCO). Virus titrations was carried out on Vero cells and expressed as 50% tissue culture infectious dose ($TCID_{50}$). After the titrations, viral stocks ($3x10^9$ $TCID_{50}$/ml)were aliquoted and stored at -70^0C and for each experiment a new vial of virus was thawed and used.

3.3. Monoclonal Antibodies

The ascites fluids, produced in pristane primed Balb/c (nu/nu) mice, were used for both *in vitro* and *in vivo* depletions . Hybridoma cells secreting T cell specific rat mAb 2.43 (Anti-CD8) and GK 1.5 (Anti-CD4) were obtained from ATCC. The concentration of antibodies were determined by an ELISA as described below.

3.4. Thymectomy and *in vivo* T cell depletions

Thymectomies were performed essentially according to the procedures described by Roubinian (1980). For the complete anesthesia of mice, a combined intraperitoneal injection of Ketamine HCl (Ketalar^R, Parke Davis, Morris Planes, NJ) at the dose of 5mg/100g body weight and methoxyflurane (Metafone^R, Pittman-Moore, Inc, Washington

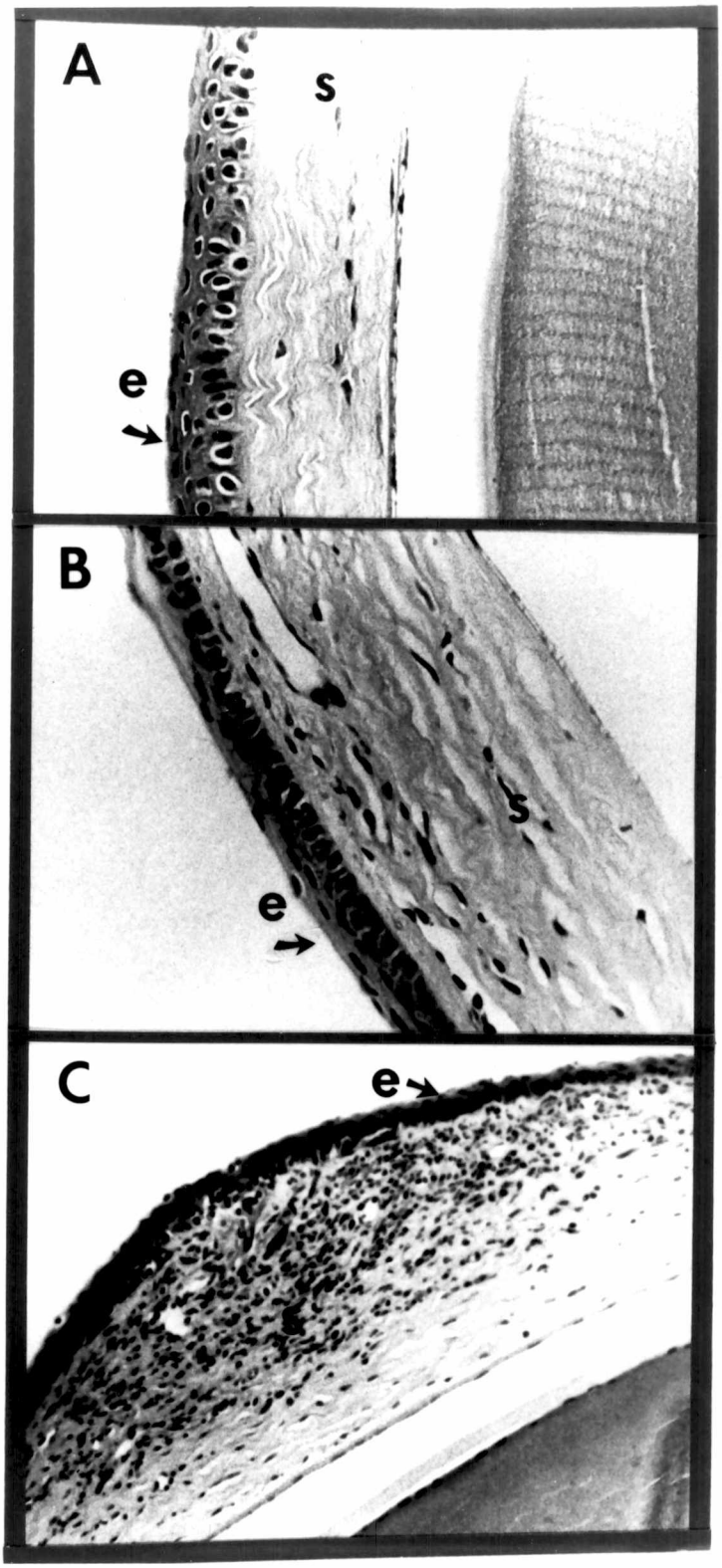
Crossing, NJ) treatment in a semi-closed ventilation unit was used. All thymectomized mice were then depleted of their circulating T cells with a cocktail mAb composed of anti-CD4 plus anti-CD8 mAb. For this purpose, 14 days post thymectomy, each mouse received 0.5 mg of GK 1.5 and 0.5 mg 2.43 mAb in 2 ml total ascites intraperitoneally.

3.5. Corneal HSV challenge

For induction of HSK, 4 ul inoculum containing $1x10^6$ ($1x10^5$ for some experiments as indicated in the text) $TCID_{50}$ HSV-1 was dropped onto the heavily scratched cornea and massaged gently for 15 seconds into the cornea using the eyelids. During corneal challenges, mice were anesthetized with the inhalant anesthetic, Methoxyflurane. (Metofane^R, Pittmann-Moore, Mondelein, IL.) This technique induces a highly reproducible stromal disease in Balb/c mice.

The clinical severity of HSK was evaluated with a slit lamp biomicroscope (Keeler Insttruments, Broomall, PA). A standard scoring system based upon the degree of opacity and cloudiness on the cornea, was used to assess the severity of the disease. According to this system, the degree of corneal inflammation varies from 0 (no visible disease on the cornea) to 5 (severe keratitis) (Newell et al. 1989a). Clinical evaluations were done in a masked fashion. Although this method of assessing the clinical severity of HSK is a frequently used system (Metcalf, 1984, Russell, 1984, Newell et al 1989a and 1989b), it is somewhat difficult to differentiate epithelial, stromal and endothelial opacities. Consequently, it seemed necessary to verify the reliability of the scoring system. Thus, in some experiments representative corneas with differing HSK scores were prepared for histopathological examination, in order to determine whether the scoring system accurately reflects the severity of stromal inflammation. The photomicrographs obtained from these sections are presented at Fig. 2. 1. As demonstrated in Fig. 2. 1, the degree of stromal inflammation increases as the biomicroscopic examination scores increase (section shown at Fig 1 A-through F represent HSK scores of 0, to 5, respectively).

Figure 2. 1. The scoring system of HSK based on biomicroscopic examination of diseased corneas gives a reliable measure of the stromal inflammation. Sections were prepared from uninfected (A) and infected corneas with a disease score of 1 thru 5 (B-F). The magnifications were 200x (A, and B) and 100x (C- F). e: corneal epithelium, s: corneal stroma.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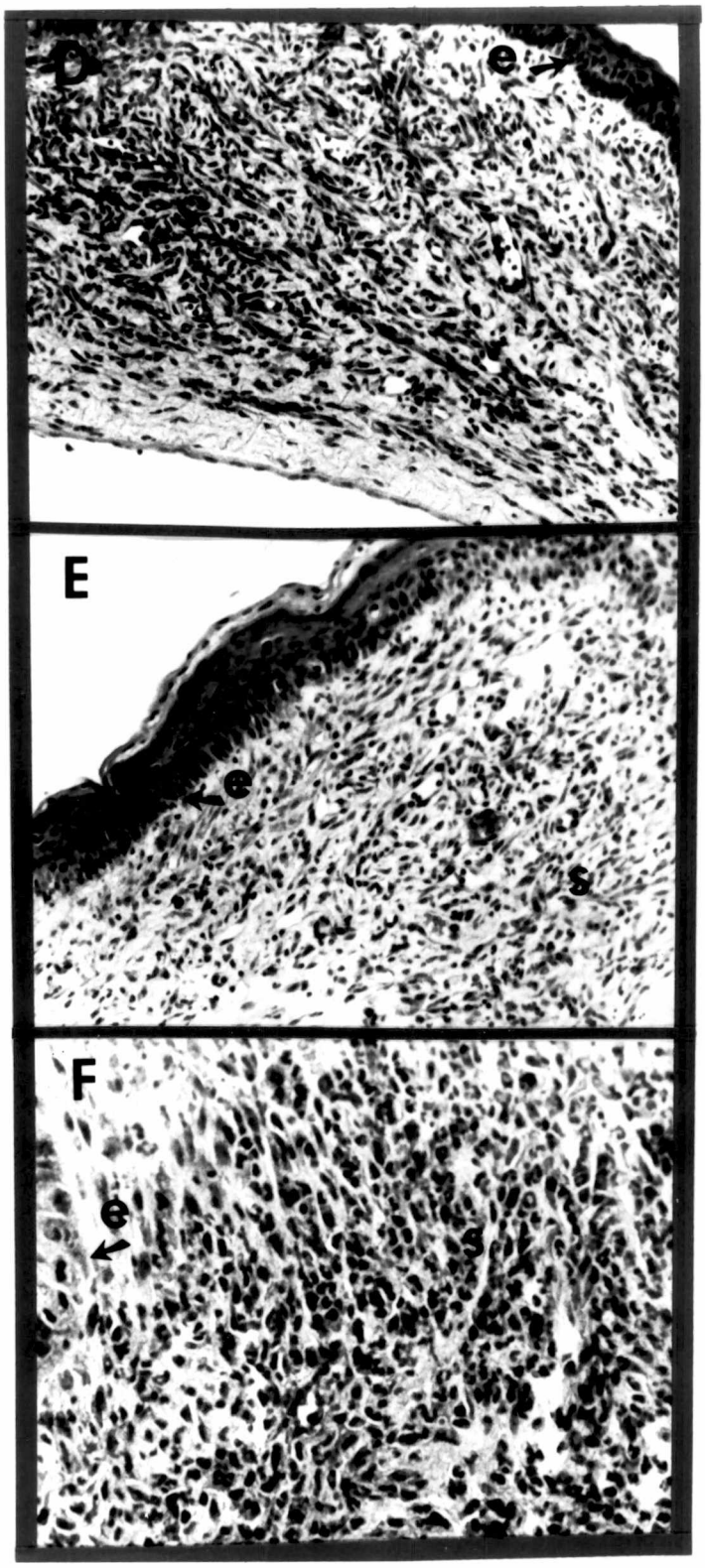
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3.6. *In vitro* lymphocyte cultures

Ten days after corneal HSV infection, cervical and retropharyngeal lymph nodes were harvested and single cell suspensions were prepared. After centrifugation, cells were resuspended in 3ml complete RPMI-1640 (GIBCO) containing 10% HI-fetal bovine serum (GIBCO), penicillin (100 U/ml), streptomycin (100 ug/ml), 7mM L-glutamine (GIBCO), 1% non-essential, 1% essential amino acid supplement (GIBCO), 10% NCTC-109 medium (GIBCO) and $5x10^{-5}$ M 2-mercaptoethanol (SIGMA Chem. Co. St Louis, MO) and infected with UV inactivated HSV-1 at a multiplicity of infection (MOI) of 2 (MOI was calculated according to virus titer before inactivation) for 30 min at $37^{o}C$. For *in vitro* culture, lymph node (LN) cells were plated in 6 well tissue culture plates (Corning Glasswork, Corning, NY.) at a cell density of $3x10^{6}$ cell/ml in complete RPMI-1640. The lymphocyte cultures were incubated for 4 days at $37^{0}C$ in a humidified atmosphere containing 5% CO_2.

3.7. *In vitro* depletions and adoptive transfer

Before adoptive transfer, T lymphocytes were separated from B lymphocytes and depleted of different subsets *in vitro* using mAb plus complement. B lymphocytes were eliminated by panning as described previously (Horohow et. al., 1987). For *in vitro* depletions, the amount of antibody required for efficient depletion were determined initiallly on Balb/c mice thymocytes. For this purpose $1x10^{7}$ thymocytes were incubated with different amounts of mAb in a total volume of 1ml for 45 min on ice. After washing 3x with PBS, cells were resuspended in rabbit complement (Low-tox Rabbit complement from Cedarlane Lab. Lim. Ontario, Canada) according to manufacturers instructions. After 45 min incubation at $37^{0}C$, the lysis of thymocytes were determined by 5% trypan blue (SIGMA) staining. The amount of mAb which lysed >90% of thymocytes was used for subsequent *in vitro* depletion experiments.After the depletions, the remaining lymphocytes were resuspended in PBS, counted, adjusted to desired concentrations, and transferred to recipient mice intraperitoneally in 1 ml PBS.

3.8. Histopathology

Sections were prepared for histopathology according to standard procedures. At the end of experiments whole eyes were fixed in 10% buffered formalin and embedded in paraffin. Six um tissue sections were stained with Hematoxylin and Eosin

3.9. Fluorescence Staining

Cells for flow cytometric analysis were prepared as follows. Splenocytes ($5x10^6$ cells/group) were centrifuged for 5 min at 250g and resuspended in total volume of 2ml with 2% paraformaldehyde (SIGMA) and fixed on ice for 30 min. Following fixation, cells were washed in PBS three times and each treatment group was aliquoted into two groups. One aliquot served as an unstained control group. The other group was stained with phycoerythrine-labelled anti-CD8 and FITC-labelled anti-CD4 antibodies (Pharmingen, San Diego, CA) for 45 min on ice. After washing extensively, cells were analysed on FACSCAN analyser (Becton-Dickinson, Mountainview, CA).

3.10. Delayed Type Hypersensitivity (DTH) reactions

Five animals from each group of mice were tested for DTH reactions at the end of the HSK experiments. For DTH challenge, 50 ul inoculum containing UV-inactivated $5x10^6$ $TCID_{50}$ HSV-1 was injected into the right rear footpad. As negative controls, 50 ul Vero cell extract were administered into the left footpads. Footpad swellings were determined 24 hr later with spring loaded calipers (Dyer Co, Lancaster, PA) and the difference between the right and left footpad was expressed in mm as a measure of DTH. Statistical significance of the difference between DTH responses of T(-) mice and other test groups were analysed by Student's *t*- test.

3.11. Enzyme-linked immunosorbent assay (ELISA)

A solid phase indirect ELISA was used to determine HSV-1 specific antibodies as described elsewhere (Martin and Rouse, 1987). Briefly, polyvinyl choloride microtiter

plates (Dynatech Lab. Alexandria, VA) were coated with purified HSV-1 and 50 ul serum samples were added to the wells and incubated for 1 hr at 37^{o}C. Following this incubation period, horseradish peroxidase-labeled goat anti-mouse antibodies (Cappel Lab.Malvern, PA) were added and the incubation was repeated. After extensive washing, the bound antibodies were detected with the substrate o-phenylenediamine (1mg/ml) and the color development was determined as optical density at 490 nm with an automated ELISA reader (Bio-Tek Inst. Burlington, VT).

The concentration of mAb present in the ascitic fluids were determined using an indirect ELISA. Microtiter plates (Dynatech Lab.) were coated overnight at 4^{0}C with 1/2000 dilutions of goat anti-rat IgG (Southern Biotech. Assoc. Inc. Birmingam, AL) prepared in carbonate buffer pH 9.6 (50ul/well). After washing the plates 3x with PBS-Tween 20 (0.05%), different dilutions of ascites fluids were added to wells (50 ul/well) and incubated 1 hr at 37^{0}C incubator. After washing, 1/1500 dilution of peroxidase conjugated goat anti-rat IgG (Jackson Immunoresearch Lab. Inc. West Grove, PA) was added to wells and the incubation was repeated. Following extensive washing, ascites antibodies were detected with the same substrate used in anti-HSV ELISA, and the results were read. Normal rat IgG (1mg/ml) (Southern Biotech. Ass. Inc.) were used as the reference sample on each plate.

4. RESULTS

4.1. HSK in T(-) mice

Previous work by several groups including our own has indicated that T cells are involved in mediating HSK (Russell et. al., 1984, Ksander and Hendrick 1987). To further evaluate the mechanism by which T cells may mediate immunopatholohy in HSK, an animal model devoid of T lymphocytes was developed. This was achived by thymectomizing mice followed by in vivo administration of both anti-CD4 and anti-CD-8 monoclonal antibodies. Such mice are described as T(-). Splenocytes from both T(-) and normal mice were analysed by flow cytometry to measure the presence of T cells. As shown at Figure 2. 2 both subpopulations of T lymphocytes were essentially eliminated in T(-) mice (44% $CD4^+$ and 14%$CD8^+$ lymphocytes in normal mice vs 2% $CD4^+$ and 0.1% $CD8^+$ cells in T(-) mice).

When injected with HSV on the cornea, T(-) mice, failed to develop signs considered typical of HSK (Fig. 2. 3). However, an acute epithelial keratitis was evident in the first week of infection and such mice showed extensive facial herpetic lesions and blepharitis. In T(-) mice, periocular herpetic lesions were a consistent feature of corneal HSV challenge and lesions usually healed within 7 days. During epithelial keratitis, the integrity of the epithelium was lost and epithelial tissues were seen as having rough surfaces when examined by biomicroscopy. Occasional mice developed encephalomyelitis. Immunocompotent mice simultaneously infected with HSV developed superficial keratitis on day 2 followed by early signs of herpetic stromal keratitis (HSK) on day 8. Such signs included an hazy appearance and opacity of the cornea. During the stromal disease, the epithelium had returned to normal, superficial lesions usually healed and epithelial surfaces regained their smooth and intact appearance.

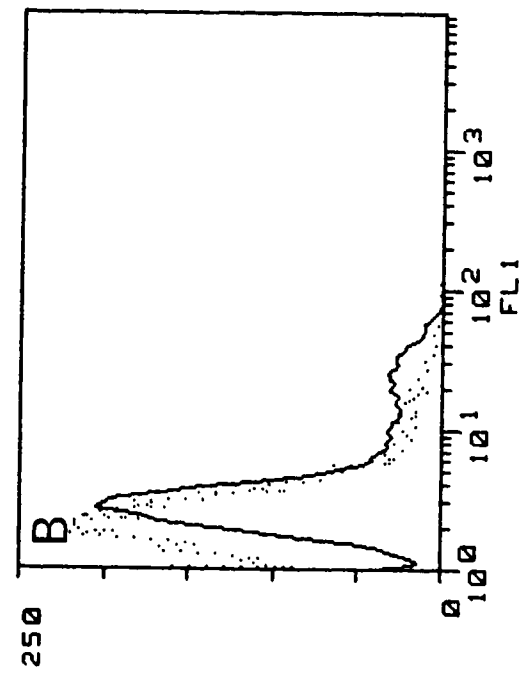
4.2. Adoptive transfer of HSV-immune cells into T(-) mice

Since T(-) mice failed to develop typical HSK, they were considered to represent a valuable model system to study the effects of adoptive transfer with various cell types. The

Figure 2. 2. Both $CD4^+$ and $CD8^+$ lymphocytes are eliminated (>95%) in T(-) mice.

A. Splenocytes from immunocompetent mice were analysed by flow cytometry.The cells were stained with FITC conjugated anti-CD4 mAb (dotted lines-44% of total lymphocytes) and PE-labelled anti-CD8 mAb (darker lines-14%).

B. After thymectomy and anti-CD4 plus anti CD8 mAb treatment, T(-) mice splenocytes were stained with anti-CD4 (solid lines-2%) and anti-CD8 (dotted lines-0.1%) antibodies. The graphs of the flow cytometry analysis were developed by overlying the images from the two fluorescein channels (FL1-FITC-anti-CD4 and FL2-PE-anti-CD8 channels). The fluorescein intensity of log 10^{-2} was used as cut off points for positive stainings.


B
250
0
10^0
10^1
10^2
10^3
FL1

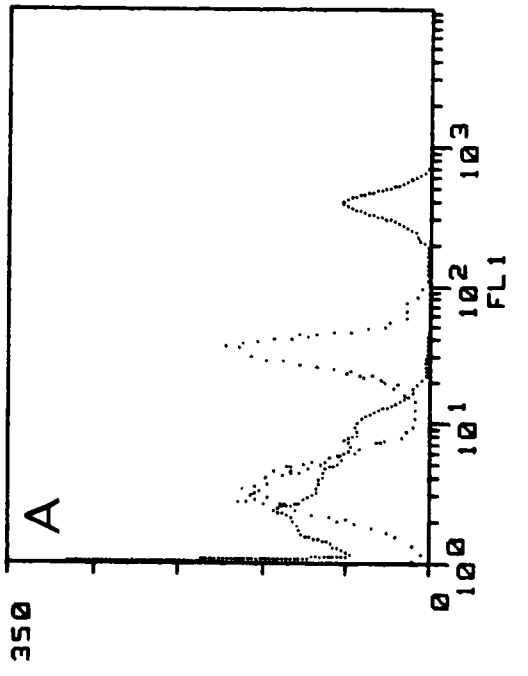
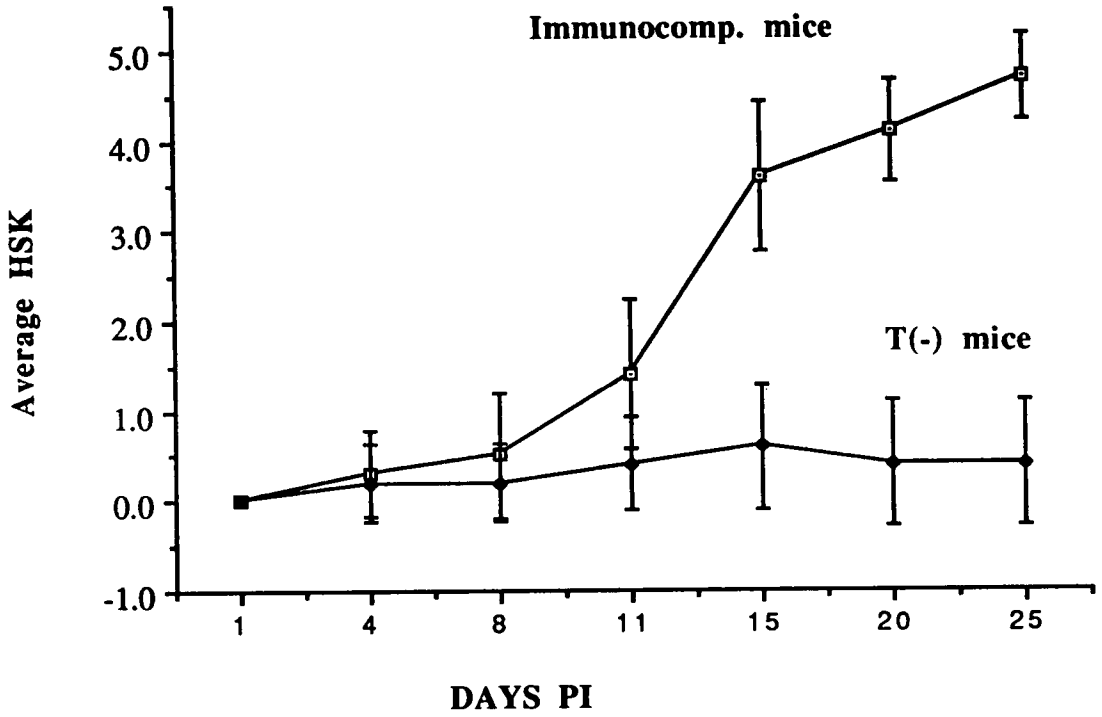

A
350
0
10^0
10^1
10^2
10^3
FL1

Figure 2. 3. The severity of HSK was reduced greatly in T(-) mice. Average HSK scores were calculated from HSV-infected T(-) (n=5) and HSV-infected immunocompetent (n=5) mice.


Immunocomp. mice
T(-) mice
Average HSK
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
1
4
8
11
15
20
25
DAYS PI

protocol used in these experiments is presented as a flow diagram in Fig. 2. 4. In the first experiment, draining LN cells from mice infected 10 days previously with HSV via the cornea were restimulated *in vitro* with UV-inactivated HSV. It is known from previous studies that this population has potent $CD4^+$ and $CD8^+$ CTL activity as measured *in vitro* , has the ability to transfer DTH and can protect mice from local and lethal infections (Rouse et. al., 1988). T(-) recipients of adoptive transfers were able to demonstrate DTH (Fig. 2. 5), a property of $CD4^+$ T cells as shown previously (Rouse et., al., 1988) and further supported by the data in Fig. 2. 5. In these experiments positive DTH reactions were evident in recipients of unfractionated cells as well as in the group that received CD8-depleted population, but these responses were less than those observed in HSV challenged non-thymectomized animals. In the recipient mice which received high numbers CD4-depleted cells, a significant level of DTH was also noted although the footpad swelling in these groups was less than in other transfer groups (unfractionated and CD8-depleted T cell transfers). Furthermore, animals given unfractionated and CD8-depleted populations developed significant levels of anti-HSV antibody responses (Table 2. 1). Antibody titers in T(-) mice were less than <1/20. The DTH and antibody experiments not only show that complete functional immunosuppression was achieved in T(-) mice but also indicate that adoptive cell transfer was successful in restoring the immune responsiveness in the T(-) mice.

The results of adoptive transfers on the development of HSK is shown in Fig. 2. 6. In mice given $5x10^6$ cells, 4 of 12 corneas had opacity scores of 2 or above and were judged to express HSK. In mice given the highest cell dose transfers ($4x10^7$), 10 of 12 corneas developed HSK. One of the corneas in the control unreconstituted T(-) mice group showed HSK with a score of 2. The most severe level of HSK noted was given a score of 4 (5 was the maximum score). With regards to the kinetics of HSK development, peak severity of lesions appeared at 11 days post infection whereas in normal mice the severity of HSK continues to progress even after 15 days of infection.

Figure 2. 4. Flow diagram for adoptive transfer experiments. The details of the procedures used are described in the Materials and Methods.

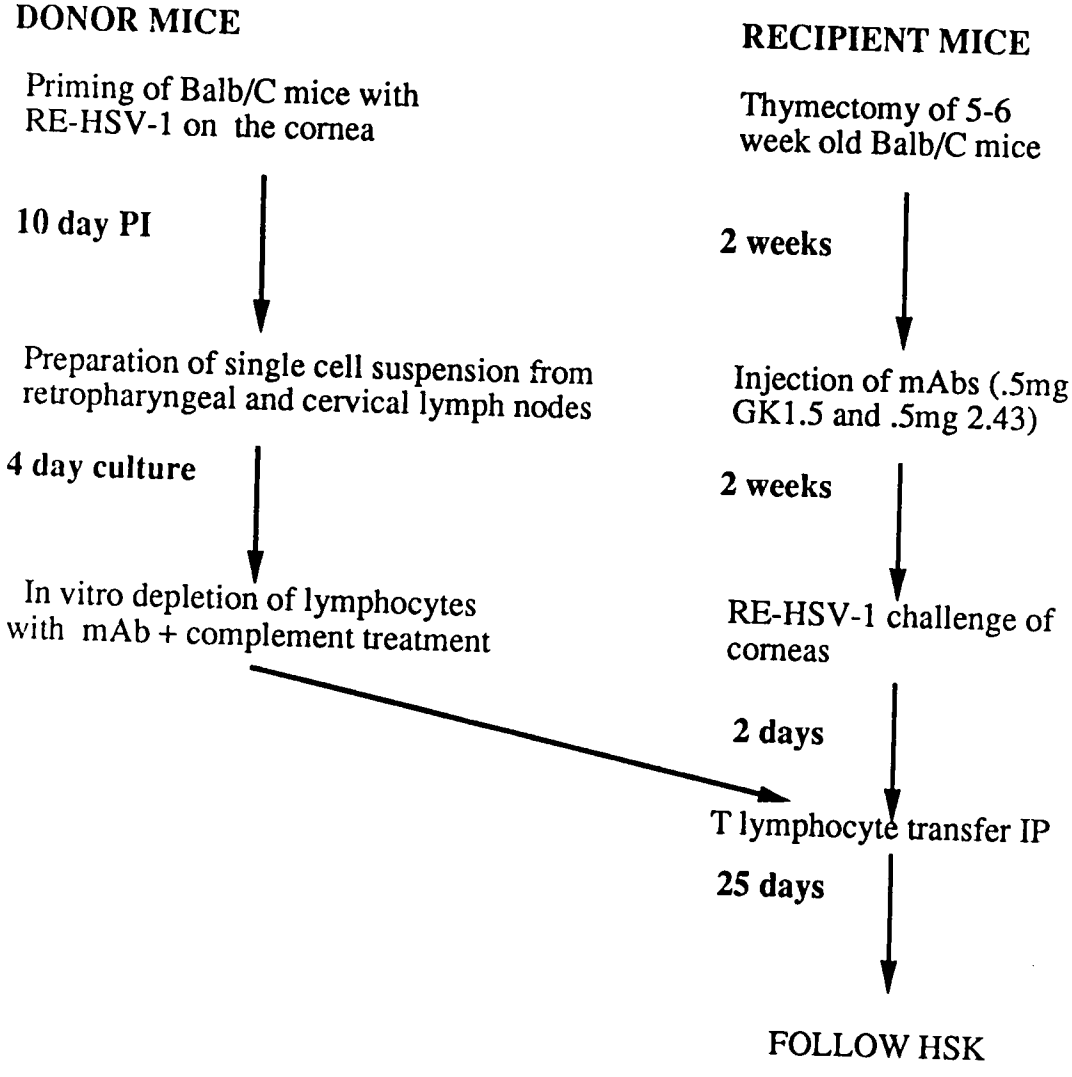

DONOR MICE
Priming of Balb/C mice with RE-HSV-1 on the cornea
10 day PI
Preparation of single cell suspension from retropharyngeal and cervical lymph nodes
4 day culture
In vitro depletion of lymphocytes with mAb + complement treatment
RECIPIENT MICE
Thymectomy of 5-6 week old Balb/C mice
2 weeks
Injection of mAbs (.5mg GK1.5 and .5mg 2.43)
2 weeks
RE-HSV-1 challenge of corneas
2 days
T lymphocyte transfer IP
25 days
FOLLOW HSK

Figure 2. 5. Thymectomy followed by T cell depletion impairs anti-HSV DTH responses in T(-) mice. The DTH responses in immunocompetent and T(-)-reconstituted with (▓) $5x10^6$, (▨) $1x10^7$, (□) $2x10^7$, (■) $4x10^7$ immune-restimulated T lymphocytes are shown. For each test group (n=5 mice), mean differences in the swellings (mm x 10^{-2}) of UV-HSV challenged and Vero cell extract-challenged footpads were determined and presented as measure of DTH responses. The statistically significant DTH responses ($p < 0.02$) were analysed with Student's *t*- test, and indicated as the cut off point on the graph.

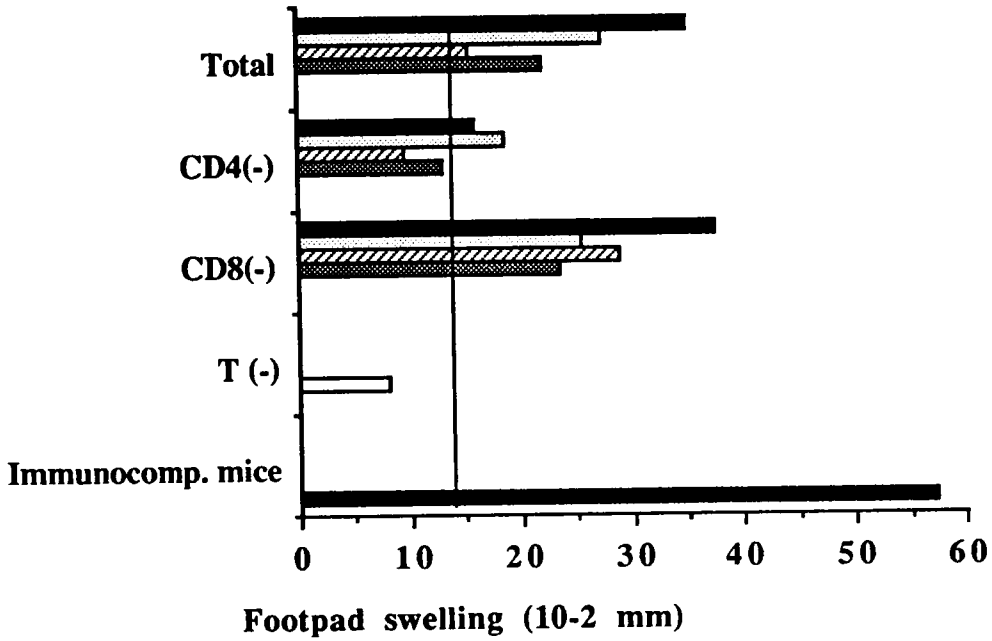

Total
CD4(-)
CD8(-)
T (-)
Immunocomp. mice
0
10
20
30
40
50
60
Footpad swelling (10-2 mm)

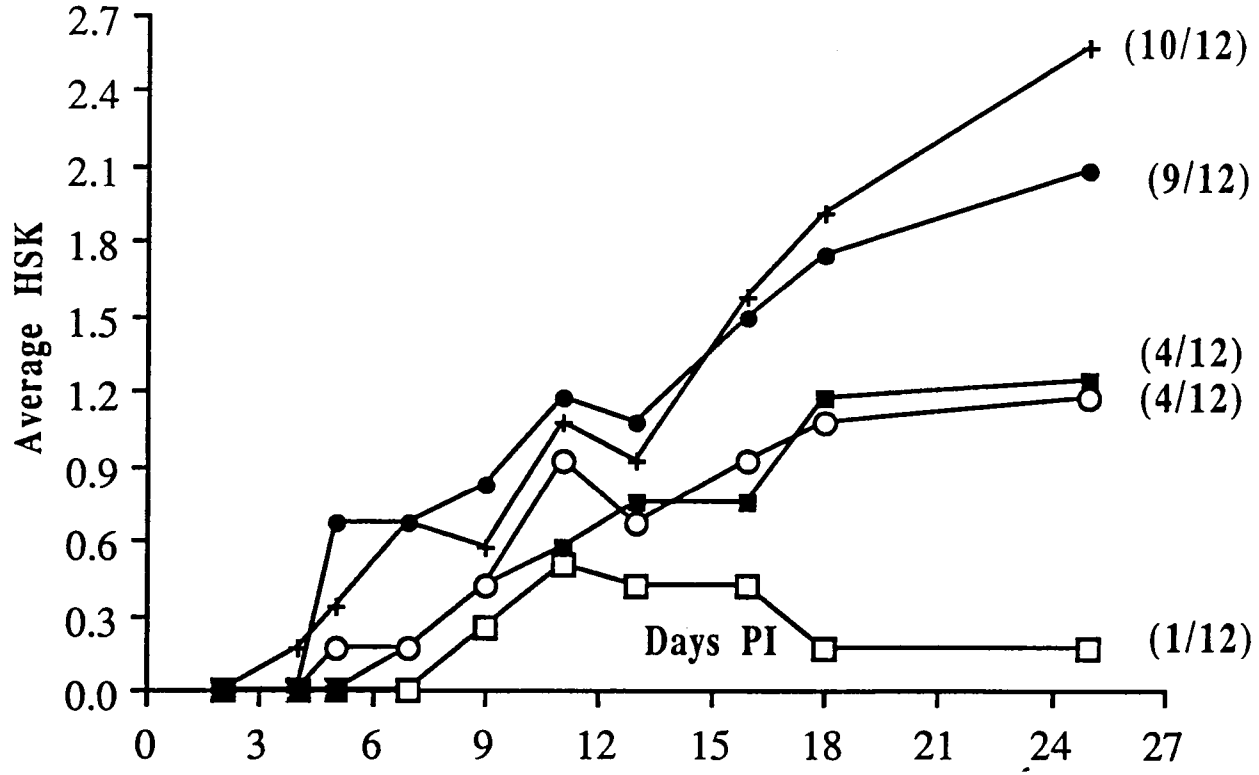
Table 2.1. Serum anti-HSV-1 antibody responses[a] of immunocompetent, T(-), and reconstituted mice.

Test Groups		Antibody Titers[b]
Normal mice		1/4000
T(-) mice		1/20
Reconstituted mice		
Total T Lymphocytes	5x107	1/160
	1x107	1/160
	2x107	1/900
	4x107	1/4000
CD8-depleted	4x107	>1/4000
CD4-depleted	4x107	<1/30

[a] Serum samples were collected 4 weeks after topical corneal challenge of mice with $1x10^6$ $TCID_{50}$ RE-strain of HSV-1. This time point coincided with the termination of HSK experiments.

[b]Antibody titers were measured in solid-phase indirect ELISA assays as described in the Materials and Methods. Positive wells were identified as wells with OD of equal or above 0.1x OD of non-immune mice serum and highest dilution of the sample with this absorbance was expressed as the antibody titer.

Figure 2. 6. Immune T lymphocytes increase the severity of HSK in T(-) mice upon adoptive transfer. Six T(-) mice/test group were reconstituted with immune-restimulated unfractionated T lymphocytes and the average HSK scores of all corneas at different time points were presented. The numbers in the parenthesis indicate the number of corneas out of 12 test corneas with HSK score of 2 or above at the end of the experiment. The test groups were: T(-) mice which did not receive T cells (□), T(-) mice reconstituted with $5x10^6$ (■), $1x10^7$ (○), $2x10^7$ (●), $4x10^7$ (✚) T cells.


Average HSK
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
0
3
6
9
12
15
18
21
24
27
Days PI
(10/12)
(9/12)
(4/12)
(4/12)
(1/12)

4.3. Nature of T cells mediating HSK in adoptive transfer model

To identify the T lymphocyte subpopulation which was responsible for transferring HSK to the recipients, T(-) mice were given *in vivo* primed and *in vitro* restimulated LN cells which had been depleted of either $CD4^+$ or $CD8^+$ populations. The amount of mAb and complement to use were previously optimized by flow cytometry. As shown in Fig. 2. 7 the transfer of HSK was mediated with unfractionated cells as well as with CD8-depleted T cell populations, but not with CD4-depleted population. In mice which received $4x10^7$ CD8-depleted T cells, 7 of 8 corneas were judged positive for HSK and the average HSK opacity scores of all corneas was 2.3. None of the mice which received CD4-depleted T cells, and mice not given cell transfers, developed HSK. Moreover, in the CD4-depleted T cell-transferred group, periocular herpetic lesions were not observed. However, such lesions were evident in all control T(-) mice.

4.4. The transfer of T lymphocytes without *in vitro* stimulation

In vitro restimulation of immune lymphocytes with virus results in a more potent immune reactive population at least as measured by *in vitro* assays (Rouse et. al., 1988). However, the *in vitro* culture conditions could affect the homing characteristics of cells and consequently could reduce the ability of transfers to induce HSK in the recipient mice. Experiments were therefore performed in which immune or naive LN cells were transferred to recipient mice without an additional *in vitro* culture step. The results of one experiment are shown in Fig. 2. 8. With such an approach, transfer was considered even more successful than was that observed with restimulated cells since the average opacity scores were greater than observed with *in vitro* restimulated cells. All corneas in animals from CD8-depleted cell recipient group developed HSK and 7 out of 10 corneas showed HSK in mice which received unfractionated cells. Whereas immune cells could transfer HSK, animals given non-immune spleen cells failed to develop stromal lesions during the observation period.

In a further study, the question of whether the adoptive cell transfers per se could

Figure 2. 7. Immune-restimulated CD8-depleted and unfractionated but not CD4-depleted T lymphocytes potentiate HSK in T(-) recipient mice. A group of 4 T(-) mice in each test group were reconstituted with no T cells (■), $4x10^7$ total unfractionated (✚), $5x10^6$ CD8-depleted (❑), $1x10^7$ CD8-depleted(●), $2x10^7$ CD8-depleted(○), $4x10^7$ CD8-depleted (◆), $1x10^7$ CD4-depleted (▲), $4x10^7$ CD4-depleted (△) T lymphocytes. Average HSK scores of all corneas at different time points were presented. The numbers in the parenthesis indicate the number of corneas with HSK score of 2 or above at the end of the experiment.

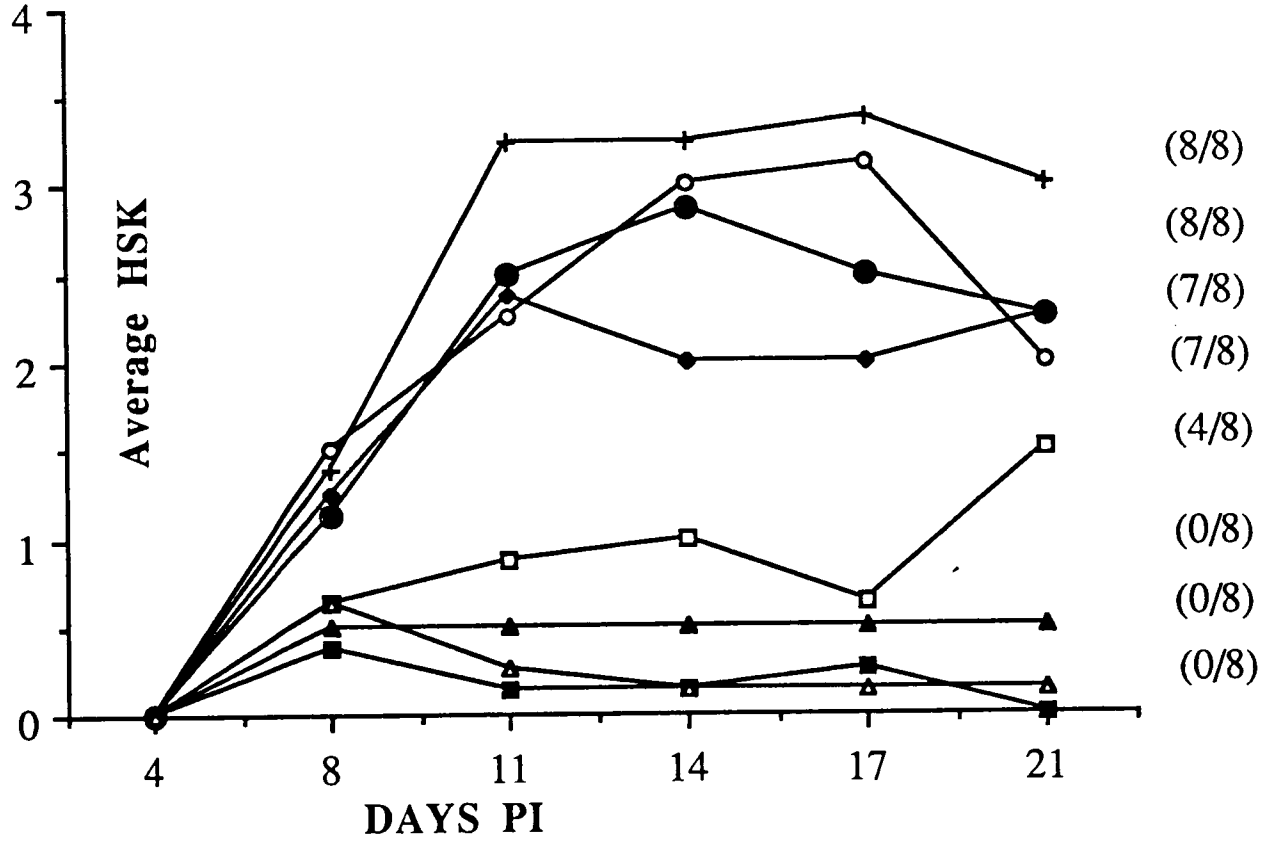
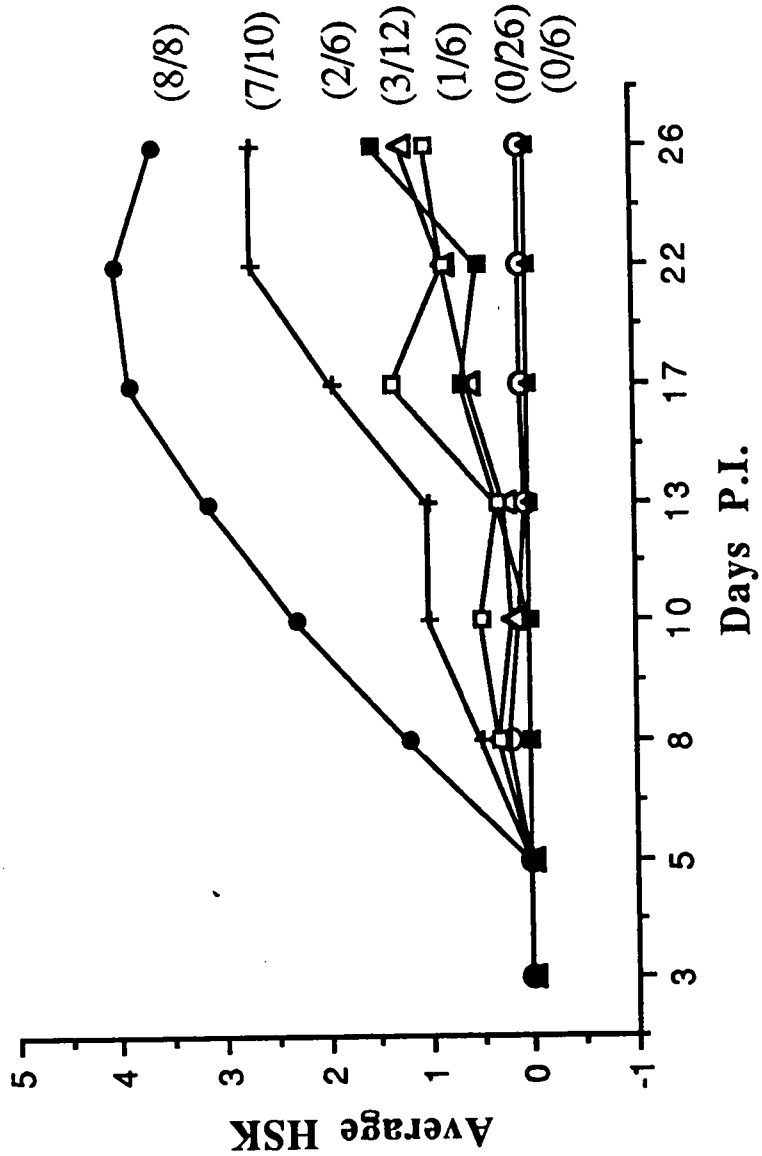

4
3
2
1
0
Average HSK
4
8
11
14
17
21
DAYS PI
(8/8)
(8/8)
(7/8)
(7/8)
(4/8)
(0/8)
(0/8)
(0/8)

Figure 2. 8. *In vitro* restimulation of immune lymphocytes is not required for adoptive transfer of HSK to T(-) mice. A group of 6 T(-) mice were reconstituted with $4x10^7$ immune CD8-depleted LN cells (●), immune-unfractionated LN cells (✚), immune CD4-depleted LN cells (▲), naive CD8-depleted splenocytes (❑), naive unfractionated splenocytes (■), naive CD4-depleted splenocytes (▲), or no (○) T cells. In no T cell transferred group, 13 mice were used. Average HSK scores were presented. The numbers in the parenthesis indicate the number of corneas with HSK scores of 2 or above at the end of the experiment.


(8/8)
(7/10)
(2/6)
(3/12)
(1/6)
(0/26)
(0/6)
Average HSK
5
4
3
2
1
0
-1
3
5
8
10
13
17
22
26
Days P.I.

exert an immunopathological response in the absence of corneal viral challenge was investigated. Thus, immune CD8-depleted T cells, seemingly the sole immunopathogenic T cell population in our model, were transferred to T(-) mice which were either mock or HSV infected previously. The challenge dose of RE-HSV for the induction of HSK was reduced to $1x10^5$ $TCID_{50}$ in this experiment in order to prevent the periocular herpetic lesions and encephalomyelitis in T(-) mice. The results of this study are presented in Table 2. 2. Herpetic stromal keratitis did not develop in either mock infected group (immunocompetent Balb/c, and T(-)-reconstituted mice). While both HSV-infected groups (immunocompetent, and T(-)-reconstituted) showed HSK with mean scores of >3 by day 11, no disease developed in T(-) mice which did not receive immune lymphocytes. A significant reduction in the severity of facial lesions in T(-) mice was noted. We interpret these data to mean that HSV infection of the cornea is required for the development of stromal pathology.

4.5. Evidence of adoptive transfer of HSK as studied by histopathology

Previous experiments have shown that unfractionated and CD8-depleted T lymphocytes isolated from draining LN of mice with HSK could transfer the disease to T(-) mice as detected by biomicroscopy. To confirm that the lesions indeed represented typical HSK, the histopathology of HSK developing in T(-)-reconstituted mice was compared to that observed in virus infected immunocompetent mice. Examinations were made on several occasions from day 1 until day 18 post infection. Inflammatory changes in the corneal stromal of HSV infected T(-)-reconstituted mice were first noted at day 9 post infection. The lesions appeared as a collection of inflammatory cells throughout all layers of the stroma. The overwhelming majority of these cells were neutrophils but low numbers of lymphocytes and macrophages were also observed. In HSV-infected immunocompetent mice, similar changes occurred in the corneal stroma at day 9 post infection. Again, the neutrophils were the predominant inflammatory cell type in this group. However, the number of cells found in the stroma of immunocompotent mice was lower than that of T(-)-reconstituted mice. At days 12 (Fig. 2. 9) and 18, in both HSV-infected immunocompetent (Fig 2. 9 A and C), and HSV-infected T(-)-reconstituted mice (Fig. 2. 9 B and D), the

Table 2. 2. Adoptive transfer of CD8-depleted T lymphocytes by itself without corneal HSV-challenge does not potentiate HSK[a].

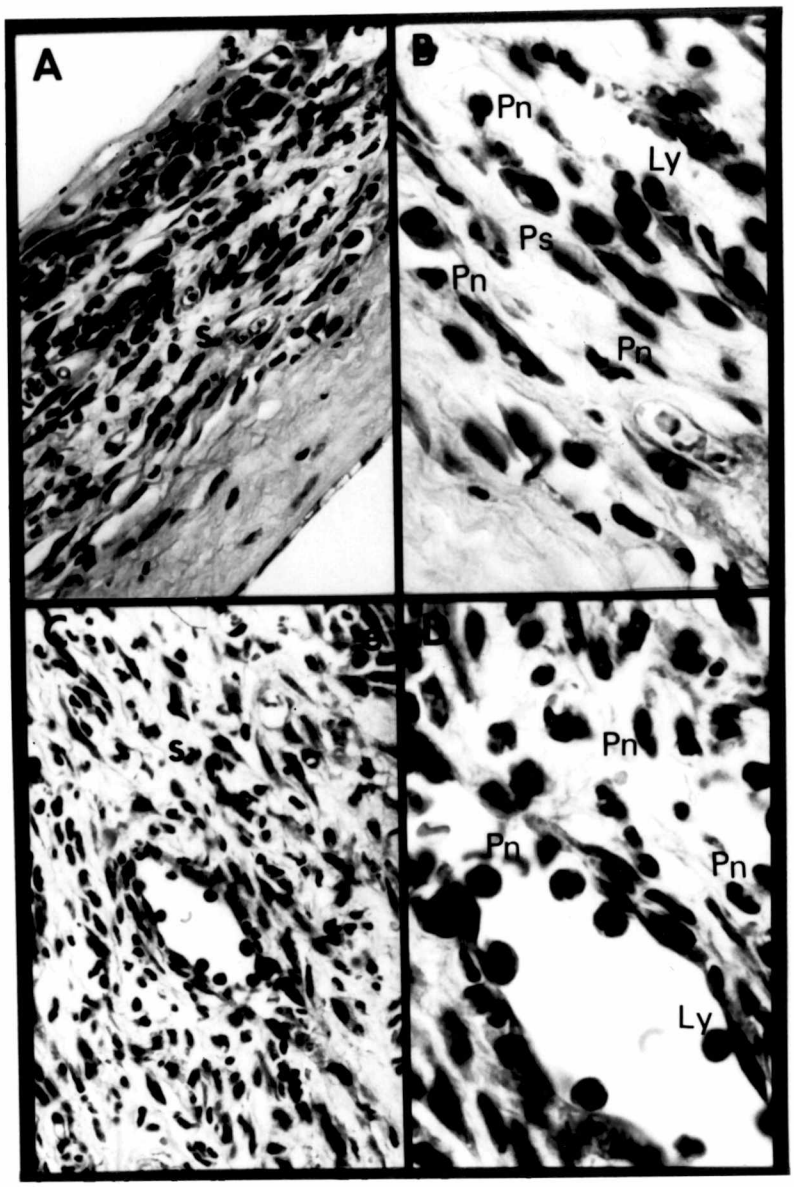
	Immunocompetent mice		T(-)-reconstituted[b]		T(-)-unreconstituted
Days PI	Mock	HSV	Mock	HSV	HSV
6	0.00[c]	1.00	0.00	0.15	0.00
9	0.00	1.81	0.00	0.18	0.00
13	0.00	2.93	0.00	3.28	0.17
18	0.00	2.70	0.00	4.25	0.25

[a] Six mice for each test group were either mock or HSV infected with the infection dose of $1x10^5$ $TCID_{50}$ and HSK was monitored during the next 18 days.

[b] T(-) mice were reconstituted with $4x10^7$ unstimulated immune LN CD8-depleted T lymphocytes at day post infection.

[c] The numbers represents the average HSK scores of the six mice obtained from biomicroscopic examination.

Figure 2. 9. Herpetic stromal keratitis in T(-)-reconstituted ($4x10^7$ CD8-depleted immune LN cells/mouse) mice (C and D) is essentially similar to HSK in immunocompetent mice (A and B). 12 days after corneal HSV-challenge, diseased corneas were prepared for histopathological examination. Panel B and D are the higher magnifications of the same areas shown at panel A and C, respectively. Original magnifications were 200x (A and C) and 600x (B and D). e: corneal epithelium, s: corneal stroma, Pn: polymorphonuclear leucocyte, Ly: lymphocyte, Ps: plasma cell.


A
B
Pn
Ly
Ps
Pn
Pn
Ps
C
Pn
Pn
Pn
Ly

the inflammatory cell types remained the same (Fig. 2. 9 C and D). At all test points, the severity of HSK in HSV-infected T(-)-reconstituted mice was higher than that of HSV-infected-immunocompetent mice. However, the inflammation in both groups was confined to the stroma and was mainly comprised of neutrophils. These results indicate that the HSK which developed in the adoptively transferred mice was essentially the same as the HSK which developed in immunocompetent mice.

5. DISCUSSION

The results of the present investigation add further support to the notion that corneal opacity that results from HSV infection and which is commonly a cause of blindness represents a T cell mediated inflammatory response. This was shown by using a T cell depletion model in which animals were thymectomized and residual T cells removed by *in vivo* administration of anti-CD4 and anti-CD8 mAb. Such T(-) mice only developed epithelial, but not stromal reactions, upon HSV infection *via* the corneal route. Usually animals recovered from infection, and as compared to athymic nude mice, there was less tendency for virus to disseminate about the face and spread to the brain to cause fatal encephalitis. T cells, specifically $CD4^+$ T lymphocytes, were implicated as mediating the stromal pathology since the adoptive transfer of immune cells to T(-) mice led to the development of stromal lesions that were indistinguishable, as judged by biomicroscopy and histopathology, to those that occurred in immunocompetent mice following corneal infection with the same virus strain. Success at transferring disease was only achieved with HSV immune lymphocytes and rather large numbers of cells were necessary. Both observations may reflect the fact that a few of the adoptively transferred cells gained access to ocular tissues. This has been the case for another ocular immunopathological disease, experimental autoimmune uveitis. Accordingly, most of the transferred lymphocytes following intraperitoneal adoptive transfer migrate to non-ocular tissues (Palestine et. al., 1986). However, a small but significant number of cells are able to home uveal tract and to start an imunopathological reaction (Palestine et al., 1986). We have yet to formally show that any of the adoptively administered cells appear in the stroma. However, upon immunohistochemical examination, both $CD4^+$ and Ia^+ cells could be demonstrated in the corneal stroma of immunocompetent mice (Part III). Neither cell type could be shown in the corneal stroma of uninfected animals.

The adoptive transfer system described in this study should help to clarify how aspects of immunity contribute to stromal keratitis. Most would agree that HSK represents an immunopathological disease (Metcalf and Kaufman, 1976, Rouse 1985, Ksander and

Hedricks, 1987) but the nature of T cells involved, how they function, and the part played by humoral antibody remains unclear. Moreover, it is entirely possible that multiple mechanisms are operating simultaneously. Our results clearly showed an essential involvement of $CD4^+$ T lymphocytes, but not $CD8^+$ cells. In contrast, others have suggested that the latter subset causes HSK (Hendricks et. al., 1989). It is possible that our failure to show a pathological role for $CD8^+$ T cells occured because the adoptively transferred $CD8^+$ T cells lacked the homing characteristics to gain access to the eye. Should $CD8^+$ T cells be demonstrated in HSK, it s likely that their function is to provide protection rather than contribute pathology. Accordingly, in previous experiments that evaluated the effects of *in vivo* depletion of $CD8^+$ T cells in HSK, an exaggerated form of the disease was observed (Newell et. al., 1989a , and 1989b). Our current working hypothesis is that $CD4^+$ lymphocytes mediate HSK by reacting some as yet to be defined viral antigens, that is probably expressed on Ia^+ Langerhans cells or resident cells expressing Ia as result of the virus infection (Mc Bride et. al., 1989). Langerhans cells are known to rapidly invade the central cornea from the limbus following HSV infection (Lewkowicz-Moss et. al., 1987). The extend of the inflammatory reaction may be limited by $CD8^+$ T cells although how this occur is unclear. Thus, $CD8^+$ cells could be down regulating $CD4^+$ function (suppressor activity) or may serve to diminish the inflammatory response by destroying target antigen expressing cells.

We currently favor the notion that HSK represents a DTH response and that several cytokines release from $CD4^+$ T cells are involved. In fact, a reduction in the severity of of HSK has been noted in animals treated with anti-interferon antibodies (Appendix, I and II). The most troubling observation not consistent with the DTH hypothesis is the fact that the most prominent cell type present during the most stages of HSK is the netrophil (Fig. 2. 9 B and D, Meyers-Elliot and Chitjian, 1981). The presence of such cells may imply that tissue necrosis is occurring or that complement is being activated such as would result following antigen-antibody complex deposition. Indeed, some investigators have suggested

that susceptibility to HSK is correlated with the IgH genotype possibly implying that antibody is in some way associated with the HSK reaction (Foster, et. al., 1986, 1991). It is also conceivable that the neutrophils are responding to tissue necrosis that results from $CD4^+$ T cells exerting a cytotoxic response against virus infected or viral antigen presenting cells. We have recently demonstrated that such HSV-specific $CD4^+$ class-II restricted CTL occur (Kolaitis et. al., 1990) and in fact are abundantly present in in the draining LN of mice with HSK (Part III). It is also possible that $CD4^+$ T cells could cause disease by a non-cytolytic mechanism of physically detaching stromal cells from their surroundings as speculated elsewhere (Abrams and Russell, 1991). Such degenerated cells could be releasing factors that are chemotactic mainly to neutrophils. Clearly more experiments are required to resolve the issue of how $CD4^+$ T cells mediate pathology in HSK. We contend the system described herein should be valuble to clarify the role of various mechanisms. It could be that such information will lead to the development of modalities useful for the control of a common disease.

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PART III:

MHC-II RESTRICTED, $CD4^+$ CYTOTOXIC T LYMPHOCYTES (CTL) SPECIFIC FOR HERPES SIMPLEX VIRUS-1: IMPLICATIONS FOR THE DEVELOPMENT OF HERPETIC STROMAL KERATITIS (HSK) IN MICE.

1. ABSTRACT

An increasing number of reports in the literature ascribe an immunopathological role for $CD4^+$ lymphocytes in the development of HSK in murine models. It has been established that $CD4^+$ lymphocytes are critically involved in the generation of anti-viral antibody and in delayed type hypersensitivity responses (DTH). However, the exact immunopathological mechanism(s) utilized by $CD4^+$ T cells during HSK remains to be elucidated. Recently, we have demonstrated that HSV immune $CD4^+$ T lymphocytes obtained from local LN after ear pinnae and footpad priming demonstrated cytotoxic activities against MHC-Class II^+, HSV infected target cells.

In this study, we searched for the presence of such cells in the cervical and retropharyngeal LN of Balb/c mice experiencing HSK. After *in vitro* depletion of $CD4^+$ or $CD8^+$ T cells with specific monoclonal antibodies and complement treatment, the cytotoxic functions of the remaining T cell populations were assayed by using target cells expressing either MHC-Class I or both Class I and Class II. Our results showed the presence of a distinct CTL population which was $CD4^+$ and demonstrated lytic activity in a Class II restricted fashion.

Furthermore, these cells were able to develop into efficient effector CTL in the absence of $CD8^+$ T lymphocytes as assessed by *in vivo* depletion experiments. Immunohistochemical methods were also utilized to show the presence of both CD4+ lymphocytes and I-A^+ cells in the corneal tissues during HSK. These findings support the notion that direct lysis of infected Class II bearing corneal cells by $CD4^+$ CTLs might be one of the mechanisms leading to stromal immunopathology in herpetic infections.

2. INTRODUCTION

Herpetic stromal keratitis (HSK) is among the most frequent causes of permanent loss of vision in the developed countries (Hyndiuk and Glasser, 1986). The possibility of T cell-mediated immunopathological reactions as a primary cause of stromal pathology has been suggested by many investigators (Kaufman, 1976, Metcalf, 1984, and Rouse, 1985).

Whereas the resolution of primary HSK infections of the cornea is rapid and complete healing is usually observed, recurrent attacks can cause a permanent damage (Kaufman, 1976). Corneal stromal lesions are characterized by an inflammatory cell influx to the corneal layers, cloudiness, loss of keratinocytes, scar tissue formation, and eventually loss of vision (McGill and Scott 1985, and Metcalf et al 1976). Histopathological findings on the diseased corneas are reminiscent of characteristic DTH reactions. However, the mechanism(s) of pathology utilized by $CD4^+$ cells have not yet been elucidated.

A murine model of HSK has been developed to study the immunological elements involved in the disease. By using an *in vivo* T cell depletion approach, we (Newell et. al.1989a and 1989b) and others (Hendricks and Tumpey, 1990) have shown that $CD4^+$ T lymphocytes play a central role in the development of HSK. Furthermore, a significant level of DTH response in mice with the disease has been demonstrated (Newell et al 1989a). This implicates a possible involvement of DTH type responses in the progress of stromal pathology potentiated mainly by $CD4^+$ T cells. However, $CD4^+$ T lymphocytes are known to play significant roles in many immune responses such as generation of antibody, CTL, and DTH. Thus, additional effector systems of immunity might be operative during the inflammatory process. Furthermore, it has been suggested in other viral immunopathological models that one T cell subpopulation could mediate pathology by different mechanisms (Doherty 1990).

Recently, we described a CTL population against HSV which is $CD4^+$ and MHC-II restricted in its function (Kolaitis et. al. 1990). These CTL were readily demonstrable

following HSV priming *via* footpad and ear pinnea routes. Since there is considerable data supporting the hypothesis that $CD4^+$ T cells are immunopathogenic in HSK, we were interested in determining whether these lymphocytes could demonstrate a cytotoxic activity. In this study, anti-herpetic CTL activity of $CD4^+$ cells obtained from retropharyngeal and cervical LN of mice experiencing HSK was tested and the relevance of this activity in the context of HSK IP was discussed.

3. MATERIALS AND METHODS

3.1. Animals

Male Balb/c mice (Harlan Sprague Dawley, Indianapolis, IN.) at the age of 6-8 weeks were used in the study. Animals were housed at the animal facilities of the University of Tennessee, Knoxville, TN. All experimental procedures were in complete agreement with the ARVO resolution on use of animals in research.

3.2. Virus

RE and KOS strains of HSV were propagated on Vero cells grown in McCoy media (GIBCO Laboratories, Grand Island, New York, NY.) supplemented with penicillin (100 U/ml), streptomycin (100 ug/ml), and 5% heat inactivated (HI) donor calf serum (GIBCO). Virus titrations was carried out on Vero cells and expressed as 50% tissue culture infectious dose ($TCID_{50}$). Vaccinia thymidine kinase (Vacc. Tk-) negative virus (originally from B. Moss, NIH) was grown on HeLa cells and titrated as described previously (Cremer et al. 1985). After the titrations, viral stocks were aliquoted and for each experiment a new vial of virus was thawed and used.

3.3.Cell Lines

EMT.6 ($H\text{-}2d^{+}$, kindly provided by Dr. Ed Cantin, City of Hope National Medical Center, Duarte, CA.), L929 ($H\text{-}2^{k}$, American Type Culture Collection, Rockville, MD.) and SJ.1 (originated from L929 cells) were grown in DMEM (GIBCO), containing 10% HI fetal bovine serum (GIBCO), penicillin (100 U/ml), streptomycin (100 ug/ml), and 7mM L-glutamine. A.20 ($H\text{-}2^{d+}$, $I\text{-}A^{d+}$, from ATCC) lymphoma cells were maintained in complete RPMI-1640 supplemented with 10% HI-FBS, penicillin (100 U/ml), streptomycin (100 ug/ml), 7mM L-glutamine, 1% non-essential, 1% essential amino acids (GIBCO), 10% NCTC medium (GIBCO), and $5x10^{-5}$ M 2-mercaptoethanol.

3.4. Monoclonal Antibodies

Culture supernatants or ascites were used for depletions and stainings of T cells. The ascites fluids were produced in Balb/c (nu/nu) mice. Hybridoma cells secreting T cell specific mAbs TIB 107 (Anti-Thy 1.2), 2.43 (Anti-CD8), GK 1.5 (Anti-CD4), RL 172.4 (Anti-CD4) and TIB 229 (Anti-IA^d) and HB 151 (Anti-HLA-DR 5) were obtained from ATCC. All mAbs were rat immunoglobulins. The concentration of mAb present in the ascitic fluids were determined with an indirect ELISA. Polyvinyl microtiter plates (Dynatech Lab. Alexandria, VA) were coated overnight at 4°Cwith 1/2000 dilutions of goat anti-rat IgG (Southern Biotech. Assoc. Inc. Birmingam, AL.) prepared in carbonate buffer pH 9.6 (50ul/well). After washing the plates 3x with PBS-Tween 20 (0.05%), different dilutions of ascites were added to wells (50 ul/well) and incubated 1 hr at 37°C incubator. After washing, 1/1500 dilution of peroxidase conjugated goat anti-rat IgG (Jackson Immunoresearch Lab. Inc. West Grove, PA) was added to wells and the incubation was repeated. Following extensive washing, ascites antibodies were detected with the substrate o-phenylendiamine (1mg/ml) (Sigma Chem. Co. St Louis, MO) and the color developed were read in an automated ELISA reader (Bio-Tek Instruments Inc. Burlington, VT). Normal rat IgG (1mg/ml) (Southern Biotech. Ass. Inc.) were used as the reference sample on each plate.

3.5. Mouse immunizations and corneal HSV challenge

Corneal infections were accomplished on deeply anesthetized (Methoxyflurane, Metofane^R, Pittmann-Moore, Mondelein, IL.) mice after the scarification of cornea with a 27 gauge needle. After virus inoculation ($1x10^6$ $TCID_{50}$-RE-HSV in 4 ul total volume), eye lids were gently massaged over infected corneas several times and virus was allowed to be absorbed by abraded corneas for 30 seconds. This technique induces a highly reproducible stromal disease in Balb/c mice. In footpad and ear pinnae immunizations, $5x10^6$ $TCID_{50}$-KOS HSV was inoculated to each site in 50 ul PBS.

3.6. *In vitro* lymphocyte cultures

At different time points post infections, mice were sacrificed and draining LN (cervical and retropharyngeal LN following corneal challenge, and politeal and retropharyngeal LN after footpad-ear pinnae priming) were harvested and single cell suspensions were prepared. After centrifugation, cells were resuspended in a small volume of lymphocyte culture medium (complete RPMI-1640) and infected with UV inactivated HSV at a multiplicity of infection (MOI) of 2 (before inactivation titer) for 30 min at 37^0C. For *in vitro* culture, LN cells were placed in 6 well tissue culture plates (Corning Glasswork, Corning, NY.) at a cell density of $3x10^6$ cell/ml in complete RPMI-1640. The lymphocyte cultures were incubated for 4 days at 37^0C in a humidified atmosphere containing 5% CO_2.

3.7. Cytotoxicity assay

On the final day of culture, cytotoxic activities of *in vitro* stimulated lymphocytes were determined by using 4 hr ^{51}Cr-release assay. Target cells were either mock or virus infected for 6 hr (MOI of 10) in 6 well culture plates at 37^0C. During the infection period, 200 uCi sodium chromate (^{51}Cr) was added to target cells. After labelling, the target cells were washed 3 times in 1mM EDTA pH 7.0 and resuspended in complete RPMI-1640 and added onto the effector cells ($1x10^4$ target cell/well). Effector cells were collected from culture wells and washed 3 times with PBS and resuspended in complete RPMI-1640. After counting, lymphocytes were distributed into 96-well V-bottom plates (Corning) in 0.1 ml volume and diluted according to desired effector/target (E/T) cell ratios. The assay plates were centrifuged (200g for 4 min.) and placed into the incubator at 37^0C for 4 hr. After this incubation, 100 ul of culture supernatants were collected and ^{51}Cr released from lysed cells were measured in a gamma particle counter (LKB-Wallac). % Specific lysis was assesed by the following formula:

% Specific Lysis=[(Experimental Release-Spontaneus Release)/(Total Release-Spontaneus Release)]x100.

3.8. Immunohistochemistry

Sections for immunohistochemical stainings were obtained from frozen corneas. Corneas were collected from mice used in cytotoxicity assays. whole eyes were frozen immediately in dichloro-difluoromethane (Accu-freeze, Baxter Healthcare Co. McGaw Park, IL), and embedded in OCT compound (Miles Inc. Elkhard, IN). Four micron sections were first fixed with 4% paraformaldehyde (SIGMA) following which an indirect immunoperoxidase staining was performed. Primary antibodies used were similar to those used in cytotoxicity assays. An avidin-biotin based anti-rat immunoglobulin immunoperoxidase kit (Stravigen, Immunohistology kit, Biogenex Lab. San Ramon, CA) was utilized to visualize cells in the sections.

3.9. T lymphocyte depletions

For *in vivo* depletions, two consecutive injections of ascites fluids were done *via* intraperitonel route at days -2 and 0 relative to the corneal primings. 0.5mg mAb in 0.2ml total volume was injected per mouse for each injection. For *in vitro* depletions, the amount of antibody required for efficient depletion were determined initiallly on Balb/c mice thymocytes. For this purpose $1x10^7$ thymocytes were incubated with different amount of mAbs in total volume of 1ml for 45 min on ice. After washing 3x with PBS, cells were resuspended in rabbit complement (Low-tox Rabbit complement from Cedarlane Lab. Lim. Ontario, Canada) according to manufacturers instructions. After 45 min incubation at 37^0C, the lysis of thymocytes were determined by trypan blue staining. The amount of mAbs which lysed >90% of thymocytes were used in subsequent *in vitro* depletion experiments.

3.10. Fluorescence Staining

Cells for flow cytometry analysis were prepared as follows. After depletions, $5x10^6$ cells were centrifuged for 5 min at 250g and resuspended in 2ml with 2%

paraformaldehyde, and fixed on ice for 30 min. Following fixation, cells were washed in PBS three times and each treatment group was aliquoted into two groups. One aliquot was used in as an unstained control group. The other group was stained with phycoerythrine-labelled anti-CD8 and FITC-labelled anti-CD4 antibodies (Pharmingen, San Diego, CA) for 45 min on ice. After washing extensively, cells were analysed on FACSCAN analyser (Becton-Dickinson, Mountainview, CA).

3.11. Statistical Analysis

The difference in the % specific lysis of mock and virus infected targets by *in vitro* and *in vivo* depleted effectors were analysed by Student's *t* - test .

4. RESULTS

4.1. CTL induction: corneal vs footpad and ear pinnae priming

The site of primary infection where the antigens are introduced to the immune system may have a profound impact on the type of immune response generated (Nash and Gell, 1983). Priming of mice with HSV *via* the footpad and ear pinnae routes is known to induce a CTL response detectable after *in vitro* culture and has been the customary approach used to study anti-HSV CTL responses (Pfizenmaier et. al., 1977). However, the extent and the nature of the cytotoxic responses induced after infection with HSV *via* the cornea, a route which results in T cell-mediated immunopathology, is ill-defined. Consequently, anti-HSV cytotoxic activities of LN cells after corneal priming were determined in parallel to those obtained after footpad-ear pinnae priming. In these assays, lysis of target cells was the function of both $CD4^+$ and $CD8^+$ CTL since none of the subpopulations were depleted. Five days after infection, local LN cells were collected, restimulated with inactivated virus for 3 days, and their cytolytic activities measured against a panel of target cells which included two MHC-compatible HSV-infected targets (A.20 and EMT.6). As shown at Fig. 3. 1 A, the corneal route of infection induced a notable anti-HSV CTL response which lysed both HSV infected MHC-compatible target cells,but not uninfected syngeneic or HSV-infected allogeneic cells. Compared to the footpad-ear pinnae route, corneal priming was more effective in inducing CTL, although, at some experiments, the difference between the two routes was marginal.

Corneal priming of Balb/c mice normally results in stromal disease which is first evident around 7-8 days post infection and starts to reach its peak severity by 10 days. As shown at Fig. 3. 1 B, when CTL responses were measured at the time of peak HSK reactions, vigorous CTL responses were detected in the local draining LN.

4.2. $CD4^+$ CTL response during HSK

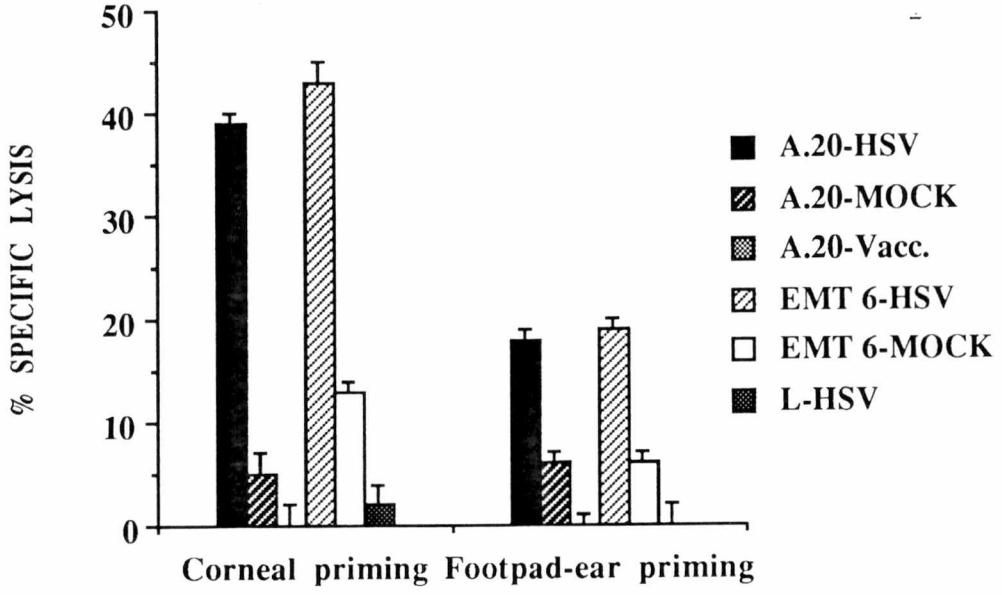
The previous experiments indicate that the corneal route of inoculation of virus induces a CTL response demonstrable as early as 5 days post infection and present in the

Figure 3. 1. Anti-HSV CTL induction following corneal and foot pad ear pinnae priming. In both panels,the results of only 50:1 E/T ratio lysis were presented.

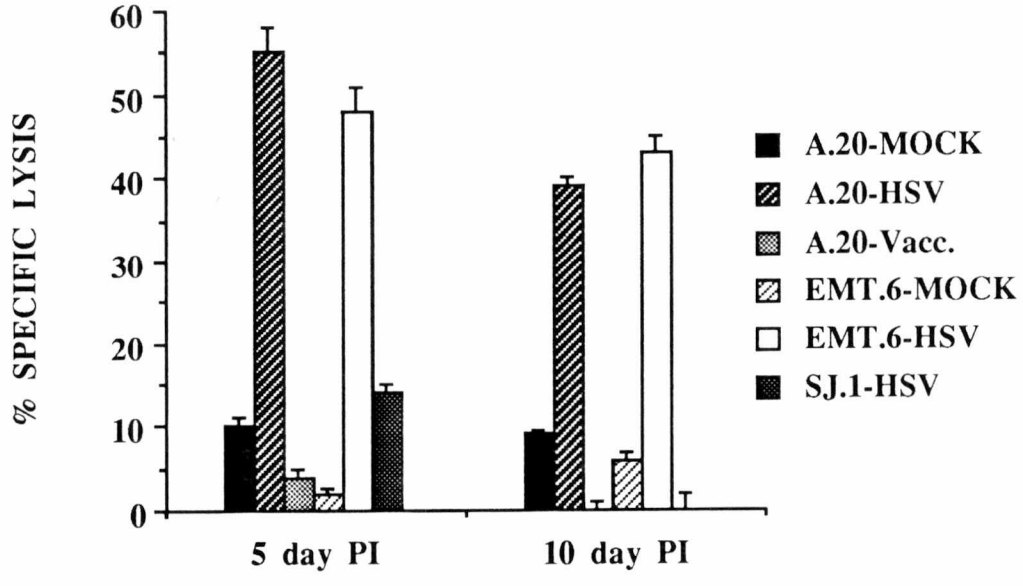
A. Cytotoxic activities of draining LN cells, 5 days after priming *via* the two routes.

B. Cytotoxicity of LN cells collected 5 and 10 days post corneal infection. All lymphocytes were stimulated *in vitro* for 4 days and CTL assays were performed without T cell depletion

A


% SPECIFIC LYSIS
50
40
30
20
10
0
A.20-HSV
A.20-MOCK
A.20-Vacc.
EMT 6-HSV
EMT 6-MOCK
L-HSV
Corneal priming
Footpad-ear priming

B


% SPECIFIC LYSIS
60
50
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A.20-MOCK
A.20-HSV
A.20-Vacc.
EMT.6-MOCK
EMT.6-HSV
SJ.1-HSV
5 day PI
10 day PI

local LN during the clinical phase of the disease. In those assays, two syngeneic target cells were used which provided us with means to study the activity of both MHC-Class-I and Class-II restricted CTL. Accordingly, A.20 lymphoma cells express both MHC-Class-I and -II molecules whereas EMT.6 cells have only MHC-Class-I antigens on the surface. By using the two different target cells and simultanously depleting T cell subsets, it was possible to identify the contribution of either Class-I or Class-II restricted CTL in the overall cytotoxic activity. Experiments were done following either *in vivo* or *in vitro* T cell subset depletion. The effectiveness of depletions was monitored by flow cytometry. Shown in Fig.3. 2 are the results of *in vivo* depletion experiments where 2.43 treatment of mice eliminated $CD8^+$ T cells completely (13 % in HB 151 group vs <1% in 2.43 treated group). In addition, the results of *in vitro* depletions demonstrated that cell populations obtained were completely devoid of one (Fig. 3. 3B) or the other subpopulations (Fig. 3. 3C). Furthermore, a cell population was produced which lacked both $CD4^+$ and $CD8^+$ cells (Fig. 3. 3D).

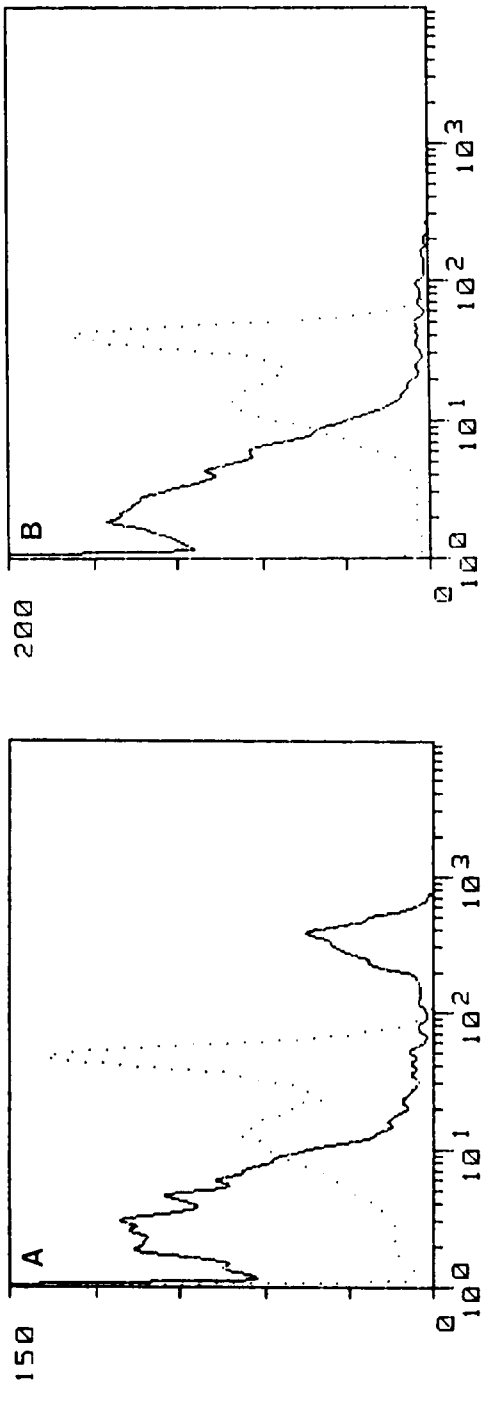
After both *in vitro* and *in vivo* depletions, all populations were tested for CTL activity against a panel of targets. If draining LN of Balb/c mice with HSK still contain $CD4^+$ CTL, after the depletion of $CD8^+$ T lymphocytes, then only HSV-infected A.20, and not infected-EMT.6, cells should be subject to lysis. The results shown in Table 3. 1 confirm this prediction. Anti-CD8 mAb plus complement treatment abolished the specific lysis of HSV-infected EMT.6 cells but effects of such treatment on A.20 lysis was only marginal. Similar patterns of responses were noted in all three experiments. If cell populations were depleted of both $CD4^+$ and $CD8^+$ T cells (i.e. anti-Thy 1.2 and anti-CD4 mAb+anti-CD8 mAb groups), then none of the targets were lysed. We interprete these results to indicate that lytic activity on A.20 target cells was exerted by two distinct T lymphocyte subsets namely $CD4^+$ and $CD8^+$ cells, whereas the lysis of EMT.6 cells was mediated only by $CD8^+$ CTL.

It was deemed of particular interest to measure the presence of $CD4^+$ CTL after *in vivo* depletion of mice with HSK since this putative immunopathological disease may be

Figure 3. 2. The results of FACS analysis. Effects of *in vivo* anti-CD8 mAb (2.43) treatment on T lymphocyte populations.

A. After HB 151 (negative control) mAb administration.

B. After 2.43 (anti-CD8) mAb treatment. Lymph node cells were stained with anti-CD4 (dotted lines) and anti-CD8 (solid lines) antibodies. The graphs in all FACS analysis experiments were developed by overlying the images from two fluorescein channels (FL1-FITC-anti-CD4and FL2-PE-anti-CD8 channels). The fluorescein intensity of log 10^{-2} was used as cut off points for positive stainings.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B
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10^1
10^2
10^3

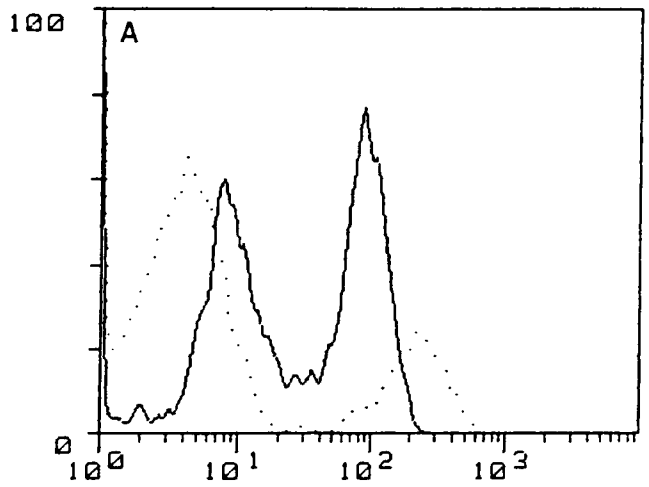
Figure 3. 3. The results of flow cytometry analysis performed following *in vitro* depletions.

A. Complement treated group. $CD4^+$ (solid lines, 45%) and $CD8^+$ (dotted lines, 19%) cells are shown.

B. Anti-CD4 mAb+ complement treated group. $CD8^+$ (darker lines) and $CD4^+$ (dotted lines) cells represented 29 and 2% of total lymphocytes, respectively.

C. Anti-CD8 mAb+complement treated cells. $CD4^+$ (solid lines, 49%) and $CD8^+$ (dotted lines, <1%) cells are shown.

D. Anti-CD4 mAb+anti-CD8 mAbs+complement treated cells. Both $CD4^+$ (dotted lines) and $CD8^+$ (darker lines) cells were less than 1% of total cells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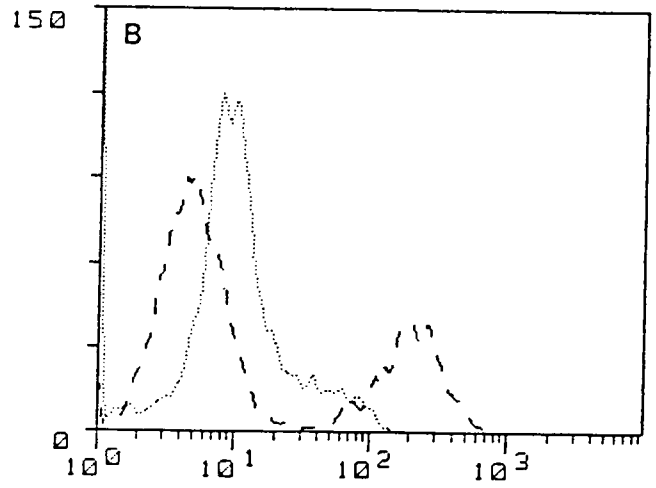
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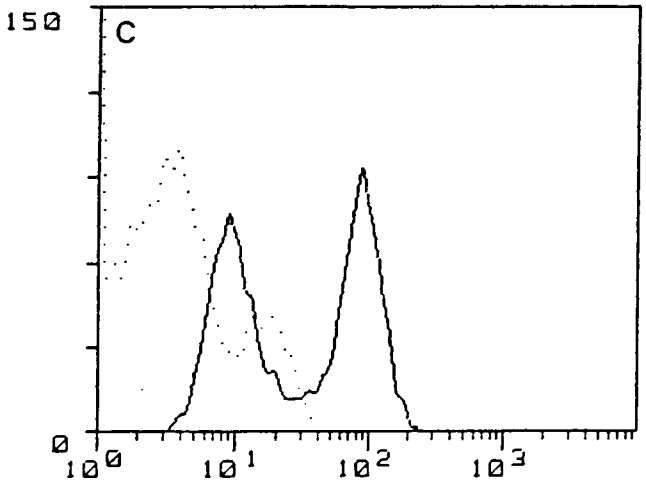
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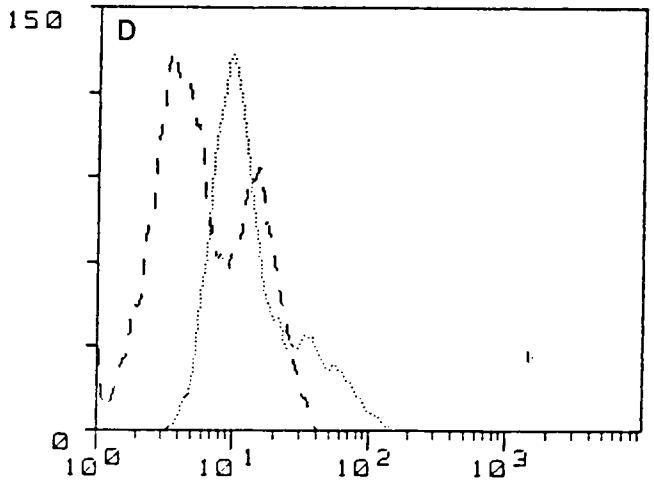
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Table 3. 1. Retropharyngeal and cervical lymph nodes of Balb/c mice experiencing HSK contain both $CD4^+$ and $CD8^+$ CTL [a].

Target Cells	A.20-MOCK	A.20-HSV	A.20-Vacc.	EMT.6-MOCK	EMT.6-HSV	L-HSV
Experiment						
c only	11 (+/-1)[b]	31 (+/-1)[c]	5 (+/-3)	11 (+/-1)	56 (+/-3)[c]	9 (+/-1)
Anti-Thy 1.2	0 (+/-1)	0 (+/-1)	0 (+/-1)	0 (+/-3)	4 (+/-1)	2 (+/-2)
Anti-CD 4	3 (+/-2)	17 (+/-1)[d]	1 (+/-1)	6 (+/-4)	52 (+/-5)[c]	7 (+/-2)
Anti-CD 8	6 (+/-1)	22 (+/-1)[c]	3 (+/-2)	9 (+/-1)	9 (+/-1)	7 (+/-1)
Experiment II						
c only	12 (+/-1)	54 (+/-1)[c]	9 (+/-2)	7 (+/-1)	47 (+/-3)[c]	11 (+/-1)
Anti-CD 4	5 (+/-1)	49 (+/-3)[c]	12 (+/-3)	8 (+/-1)	47 (+/-2)[c]	4 (+/-1)
Anti-CD 8	4 (+/-1)	23 (+/-1)[d]	7 (+/-1)	8 (+/-1)	12 (+/-2)	5 (+/-2)
Experiment III						
c only	9 (+/-2)	34 (+/-1)[c]	ND	9 (+/-2)	24 (+/-2)[d]	ND
Anti-CD 4	7 (+/-2)	21 (+/-1)[d]	ND	10 (+/-1)	26 (+/-1)[d]	ND
Anti-CD 8	11 (+/-1)	30 (+/-2)[c]	ND	9 (+/-1)	8 (+/-1)	ND
Anti-CD 4+ CD 8	4 (+/-1)	7 (+/-1)	ND	5 (+/-3)	7 (+/-1)	ND

[a]Mice were challenged on the cornea with $1x10^6$ $TCID_{50}$ RE-HSV-1, 10 days later lymph nodes were extracted, and a modified Pfizenmeir assay were set up as described in the *Materials and Methods* section.
[b]% specific lysis were determined after 4 hr ^{51}-Sodium Chromate release assay as described in the text and % lysis only at 50:1 Effector cells/Target cells ratio were presented.
[c] $p< 0.003$, [d] $p< 0.01$.

an *in vivo* expression of one or both types of CTL. The results in Fig. 3. 4 present the effects of *in vivo* depletion experiments in which local LN CTL responses were measured 10 days post infection at the height of the disease. Following *in vivo* depletion of $CD8^+$ T cells, the LN population of mice with HSK contained CTL which could lyse A.20 target cells (Fig. 3. 4 B), but not EMT.6 cells (Fig. 3. 4 E). However, the cell population from HSK mice treated with control mAb HB 151 lysed both A.20 and EMT.6 targets.

4.3. Demonstration of IA^{d+} cells and $CD4^+$ T lymphocytes in the corneal stroma

In the next series of experiments, the presence of MHC-II expressing cells in the corneal stroma was tested. Frozen sections stained with anti -IA^d, anti-Thy 1.2, and anti-CD-4 antibodies all of which were rat origin. Positively stained cells were visualized by an anti-rat-immunoglobulin based immunoperoxidase approach. The results of these experiments are summarized at Table 3. 2 and Fig. 3. 5. Uninfected corneas contained neither detectable $CD4^+$ T cells nor IA^+ cells (Fig. 3. 5 A), yet, in the inflammed areas of infected corneas, cells positive for either staining were abundant (Fig. 3. 5 B, and C).

Figure 3. 4. Cytotoxic activities of LN cells obtained from mice treated with either anti-CD8 (2.43) or negative control mAb (HB 151) 10 days after corneal infection. A panel of target cells, A.20-mock (**A**), A.20-HSV-1 (**B**), A.20-Vacc-Tk- (**C**), EMT.6-mock (**D**), EMT.6-HSV-1 (**E**), and S.J.1-HSV-1 (**F**) were used.% Specific lysis against A.20-HSV targets (**B**) by both HB 151 and 2.43 group effectors and against EMT.6-HSV targets (**E**) by HB 151 group effectors were significantly higher ($p > 0.01$) than that of against mock infected control targets in all E/T ratios as determined by Student's *t* -test.

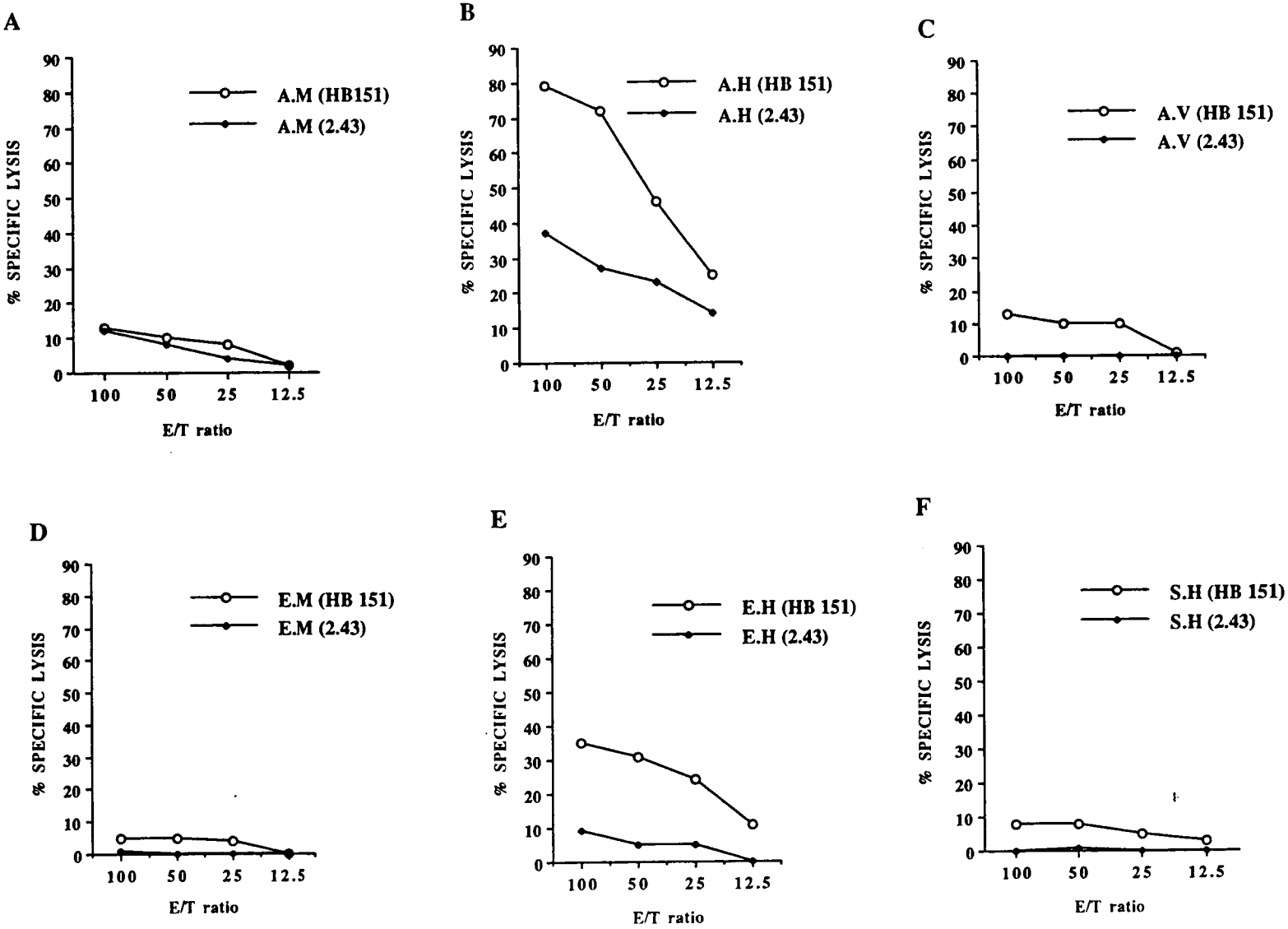
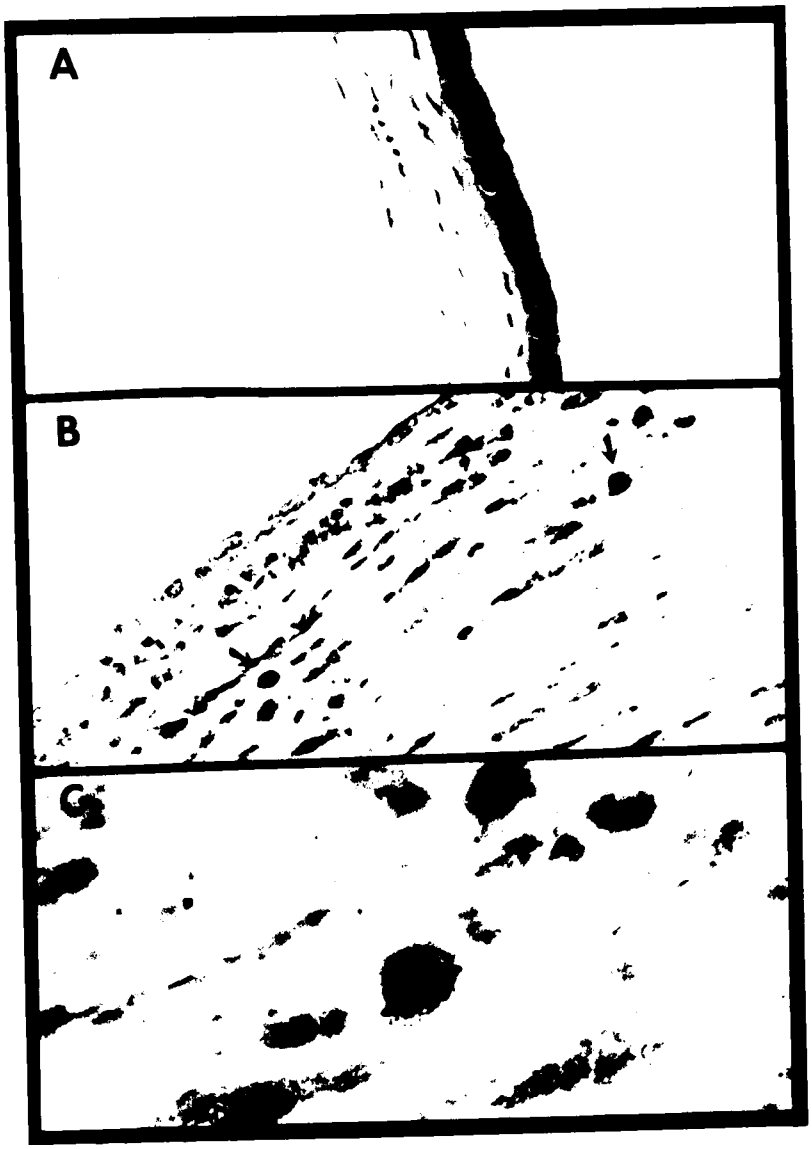

A
% SPECIFIC LYSIS
A.M (HB151)
A.M (2.43)
E/T ratio
B
A.H (HB 151)
A.H (2.43)
C
A.V (HB 151)
A.V (2.43)
D
E.M (HB 151)
E.M (2.43)
E
E.H (HB 151)
E.H (2.43)
F
S.H (HB 151)
S.H (2.43)
100
50
25
12.5

Table 3. 2. HSV infected corneal stroma contains cells which express MHC-Class II molecules on their surfaces and is infiltrated by $CD4^+$ T cells.

	mAbs used in staining			
Corneal Sections	Anti-IA^d	Anti-Thy 1.2	Anti-CD4	Anti-DR
Mock infected	(-)[a]	(-)	(-)	(-)
HSV infected	(++)	(++)	(+)	(-)

[a]Negative and positive values indicate presence or absence of the cells expressing the target molecule. Double positives indicate a high number of positively stained cells.

Figure 3. 5. Immunohistochemical staining of uninfected (**A**), and HSV-infected (**B**, and **C**). The sections were stained with anti-CD4. The original magnification were 200x (**A, B**) and 600x (**C**).


A
B
C

5. DISCUSSION

The results of this study indicate that both $CD4^+$ and $CD8^+$ CTL are present in the local LN of mice experiencing herpetic stromal disease. The cytotoxic activity of $CD4^+$ CTL is directed towards target cells expressing MHC-Class II molecules (A.20), whereas $CD8^+$ CTL appear to lyse both target cells (A.20 and EMT.6). Since almost all murine target cells used for *in vitro* cytotoxicity assays express MHC-Class I, unequivocal demonstration of $CD4^+$ CTL was received little attention in the past. By using two target cells with differential MHC expression combined with T cell depletion, we were able to demonstrate $CD4^+$ CTL in a murine model of an important human disease, HSK.

Most studies dealing with CTL in HSV murine models have focused on $CD8^+$ T cells (Martin et. al., 1989). The elucidation of the specifity and the role of such cells have been the main aims of these investigations. Accordingly, $CD8^+$ CTL were deemed necessary for the recovery of animals from high dose virus challenge, whereas $CD4^+$ T cells appeared capable of handling a low viral dose in lethality experiments (Martin et. al., 1988). However, data collected from human subjects with herpes infections suggests that anti-herpetic CTL responses are dominated by $CD4^+$ CTL, thereby assigning them an important role in the immune response against the virus (Schmid, 1988, Yasukawa and Zarling, 1984). For this reasons, our method of studying anti-herpetic CTL responses in mice might be an appropriate system to study the immunological elements of human HSV infections .

The presence of $CD4^+$ CTLs in viral infections has been reported in other viral systems. However, in most instances such findings rely on clonal cell lines (Morrison et. al., 1986, Kaplan et. al., 1984). $CD4^+$ CTL are known to be ineffective lytic cells and cultured $CD4^+$ cells on occasion have been reported to loose or to gain cytotoxic functions . This prompted some researchers to speculate on the relevance of $CD4^+$ CTL *in vivo* (Flescher and Wagner, 1986). However, the results presented are derived not from clonal

cell lines but from polyclonal assays and therefore, subscribe a relevant *in vivo* function to $CD4^+$ CTL.

A principal role for $CD4^+$ T cells in the development of HSK is becoming increasingly clear. In CD4+ T cell-depleted mice, no significant clinical symptoms of HSK is observed; in contrast the elimination of $CD8^+$ T cells does not have such effect on the progress of the disease (Newell et. al., 1989 and 1990). Thus, the results presented in Figure 3. 4 are particulartly interesting because they imply that at least some of $CD4^+$ T cells may exert a cytotoxic function on HSV infected, MHC-Class II^+ cells.

In uninfected corneas, stromal cells do not normally express MHC-II molecules. However, viral infections and subsequent inflammatory process have been shown to induce expression of both MHC-Class I and Class II antigens on many cell types (Massa et. al., 1986, and McBride et. al., 1988). Such findings were published on human corneal cells obtained from patients with recurrent ocular herpes infections. Our results also demonstrate the presence of MHC-II expressing cells in ocular tissues during HSK. However, whether these MHC-II^+ cells are actually infected with the virus, and present viral antigens in the context of their I-A molecule and consequently are subject to lysis by $CD4^+$ CTL remains to be studied. In this study, the function of $CD4^+$ T cells which infiltrate the cornea has not addressed directly. Instead, we concentrated on the activities of local LN which drain the cornea. In most *in vivo* studies, local LN cells have been the starting point for more detailed investigations (Reddehase et. al., 1986, Grau et. al., 1986, Heinzel et. al., 1989). Nevertheless, it would be of interest to isolate and be able to determine the cytotoxic activities of $CD4^+$ T cells present in the corneal stroma.

Reports on the lytic mechanisms of $CD4^+$ CTL are scarce compared to the studies on the lysis exerted by $CD8^+$ CTL (Peters et. al., 1990). Lytic molecules such as TNF-alpha and lymphotoxins are implicated in $CD4^+$ CTL lysis (Tite, 1990). Accordingly, tumor necrosis factor (TNF) secreted by $CD4^+$ CTL is the primary mediator of the lysis of influenza infected target cells since the addition of anti-TNF-alpha antibodies to the final

assay can to halt the lysis (Tite, 1990). Moreover, lysis characterized by the target cell DNA fragmentation has also reported (Ju et. al., 1990). An additional mechanism of possible tissue damage utilized by $CD4^+$ lymphocytes has also been recently reported (Abrams and Russel, 1991). Apparently, $CD4^+$ inflammatory type lymphocytes can cause target cells to detach from their extracellular surroundings without lysing the targets. Consequently, this form of tissue disruption may induce an inflammation in the area (Abrams and Russell, 1991). The precise mechanism of the potentiation of stromal inflammation in HSK by $CD4^+$ cells remains to be investigated.

$CD4^+$ T cells, in many investigative models, are associated with DTH reactions and the provision of help for antibody responses. We anticipate the possibility that a leading task of $CD4^+$ T lymphocytes in the HSK murine model may be as mediators of DTH reactions. However, this possibility does not exclude an additional mechanism of immunopathology, namely the cytotoxic activities of $CD4^+$ T cells as demonstrated in this study. The likelihood of multiple immunopathological mechanisms potentitiated by one T cell subset ($CD8^+$) in the central nervous system degeneration caused by lymphocytic choriomeningitis virus (LCMV) in mice after intracranial injections has been discussed previously (Doherty et. al., 1990). Accordingly, anti-LCMV CTL responses are the exclusive function of $CD8^+$ T cells. Furthermore, these cells play a significant role in DTH responses raised against the virus. Inflammatory exudate collected from cerebraspinal fluid of infected mice virtually lacks $CD4^+$ lymphocytes and the histopathological findings in the CNS sections reveal a massive DTH reactions during the fatal choriomeningitis. Consequently, it has been suggested that both cytotoxicity and DTH might be immunopathogenic mechanisms orchestrated by $CD8^+$ T cells (Doherty et.al., 1990). Thus, we propose a similar mechanism of immunopathology in HSK, although the relative contributions of these mechanisms to the overall development of corneal lesions are not yet definitive. The proportions of the roles played by the different effector arms of the immune system during the process of HSK immunopathology remains a major problem to be

investigated.

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PART IV:

CONCLUSIONS AND FUTURE PROSPECTS

The studies reported in this dissertation were aimed at enhancing our understanding of the immunopathology in HSK. Two main conclusions can be drawn from the results presented. Firstly, the adoptive transfer experiments described here not only strengthen the hypothesis for a $CD4^+$ T cell mediated pathology in HSK but this approach also holds promise as a useful system to further elucidate the mechanisms of immunopathology mediated by these cells. Secondly, the data related to MHC-class II restricted cytotoxicity provide another potential mechanism by which $CD4^+$ T cells may induce immunopathology in this disease.

The results of the adoptive transfer experiments indicated that $1x10^7$ CD8-depleted T lymphocytes could potentiate the disease in T(-) mice. The primary advantage of T(-) mice is that they are more resistant to encephalitis and periocular herpetic lesions than the nude mice. It is also likely that the adoptive transfer model can be improved such that it will be possible to transfer the disease with a smaller number of cells (e.g. by using potent T cell clones or a long term cultured polyclonal population).

It is clear however, that the immunopathogenic cell population to study in details is $CD4^+$ T lymphocytes at least in the Balb/c-RE-HSV model. To further study the role of $CD4^+$ T lymphocytes in HSK, one needs to consider recent data which indicate that $CD4^+$ T cells are comprised of two distinct subpopulations referred to as T_{H1} and T_{H2}, respectively. Since either of these subsets does not have an exclusive surface marker, the only differentiating characteristics is their rather distinct lymphokine secretion patterns. Accordingly, gamma-interferon is secreted by T_{H1} cells only, whereas IL-4 is secreted by T_{H2} lymphocytes. An important initial experiment is to determine the role of these lymphokines in HSK. In our current study, the attempts were made in this direction. Anti-gamma interferon and anti-IL 2-receptor antibodies seemed to reduce the severity of the disease. However, the data were not convincing. Further conclusive evidence will be needed in order to answer the question of which T_H subset is immunopathogenic in HSK. More definitive support for the role of lymphokines in the development of immunopathology may come from the analysis of the quantity of lymphokine messages

present in the disease-transferring lymphocytes, i.e. in the lymphocytes obtained from the draining lymph nodes of mice with HSK, in lymphocytes obtained from corneal stroma, or if possible in disease-transferring clones. Another lymphokine which may potentially be involved in the diease is TNF-alpha since this molecule seems to be the major lytic molecule used by $CD4^+$ CTL.

It is imperative to keep in mind that the model described here is an animal model and animal models are useful as long as they can be related to the actual human diseases. At this point data gathered from Balb/c model on $CD4^+$ T cell mediated immunopathology have not been extended to the human conditions. In human HSK, it is not yet known whether T cells mediate the disease and whether these cells are $CD4^+$. It may be premature to look for such evidence in human patients; however, attempts to relate the findings from animal models to the human disease should be planned. It is conceivable that more human oriented studies could be undertaken using SCID-hu/hu mice where one can look at the role of human immune components in the disease. It is likely that the migration of human lymphocytes in mouse tissues and the subsequent mediation of an inflammatory reaction in corneal stroma by these lymphocytes may not follow the same mechanisms as those which occur in human HSK. However, in SCID mice, it may be possible to perfom the studies described in Balb/c mice such as the depletion of T lymphocytes, and the adoptive transfer of T lymphocyes.

The adoptive transfer and *in vivo* depletion experiments indicated that $CD8^+$ T cells may actually play a protective role. This finding could be further exploited using adoptive transfer system as a possible method for intervening into the progression of HSK. Accordingly, in humans HSK develops over a long period of time and physicians often feel helpless in preventing the tragic outcome. Since, the adoptive transfer model indicates that $CD8^+$ T cells appeared to have a protective role, it may be possible to halt or to reverse the immunopathological process with $CD8^+$ lymphocytes. Thus, by administering $CD8^+$ T cells prior to HSV infection (prophylactic approach), or during the disease development (therapeutic approach), the pathological role of $CD4^+$ T cells could be

negated. Even though it may prove unrealistic to give autologous $CD8^+$ T cells to humans undergoing herpetic eye disease, further exploration in these directions may have positive implications for other areas. However, in order to perform such experiments using the adoptive transfer approach, the model must be further refined (i.e. optimum number of $CD4^+$ T cells required to induce the disease and the number of $CD8^+$ T cells to counteract the immunopathogenic activities of $CD4^+$ T cells must be definitively determined).

The role of the virus and viral antigens in the disease process seems to be minimal. However, in the absence of infection no disease develops. The implication is that there must be some level of viral antigen expression which continuosly stimulate immunopathological T lymphocytes. Moreover, it has also been suggested that HSV may not undergo complete dormant state in the corneal tissues during so called "latency" , but may be in a state of much like persistent infection where a low level of viral gene expression occur (H. Kaufman, ARVO Meeting, 1991). Newertheless, it is important to determine the viral gene expression during the HSK as studied in our model. The viral status in $CD4^+$ and $CD8^+$ T cell transferred mice may provide important clues on both the mechanism of immunopathology, and the specifity of the immunopathological cells. For example expression of a $CD8^+$ T cell epitope may implicate that $CD8^+$ T lymphocytes are reacting to the virus infected cells (removal of antigenic stimulus) rather than suppressing the activity of $CD4^+$ T cells.

Although the studies suggested in this chapter may not have immediate clinical applications, the investigations on the immunopathology of HSK could yield information on the more general aspects of viral pathogenesis.

PART V:

APPENDIX

Figure 5. 1. The mAb against gamma-interferon reduces the severity of HSK in adoptive transfer model. A group of 4 T(-) mice (11mice for no cell transfer group) were reconstituted with $4x10^7$ immune-unstimulated CD8-depleted T lymphocytes obtained as described previously. Anti-Interferon (R46A2, 1mg/mouse), anti-IL-4 (1mg/mouse) and anti-LT-TNF (TN 39, 0.3mg/mouse) antibodies were injected at 2, 7, 15, days post cell transfer intraperitoneally. The average HSK scores were presented. The numbers in the parenthesis indicate the number of corneas with the disease score of 2 or above. The main conclusion of these experiments is given in the title of this graph. However, in the present form the data do not seem consistent with the overall theme of " no disease in T(-) mice". Because 9 out of 22 corneas in T(-) group developed the disease. The possible explanations include the incomplete thymectomy or T cell depletions. The experiments should be repeated and if similar trend is obtained in terms of the effects of mAb on the development of HSK the data could be published. Another point regarding to these experiments is that the amount of antibodies used in these experiments are obtained from unrelated models (J. Immunol. 143: 266-274, J. Immunol.137: 702-707). Thus the optimization of the amount of the antibodies may be needed. Except anti-TNF secereting one, hybridomas for most of the antibodies mentioned are available from ATCC and present in the lab. The production and usage of of polyclonal anti-TNF antbodies are described elsewhere (J. Exp. Med. 170: 2097-2104, and Immunology, 69: 570-573). The dose -response experiments may be planned in immunocompetent mice and the determined dosage could be used in T(-) mice since the number of mice used in T(-) set up is at least twice as much as it would be in immunocompetent mice.

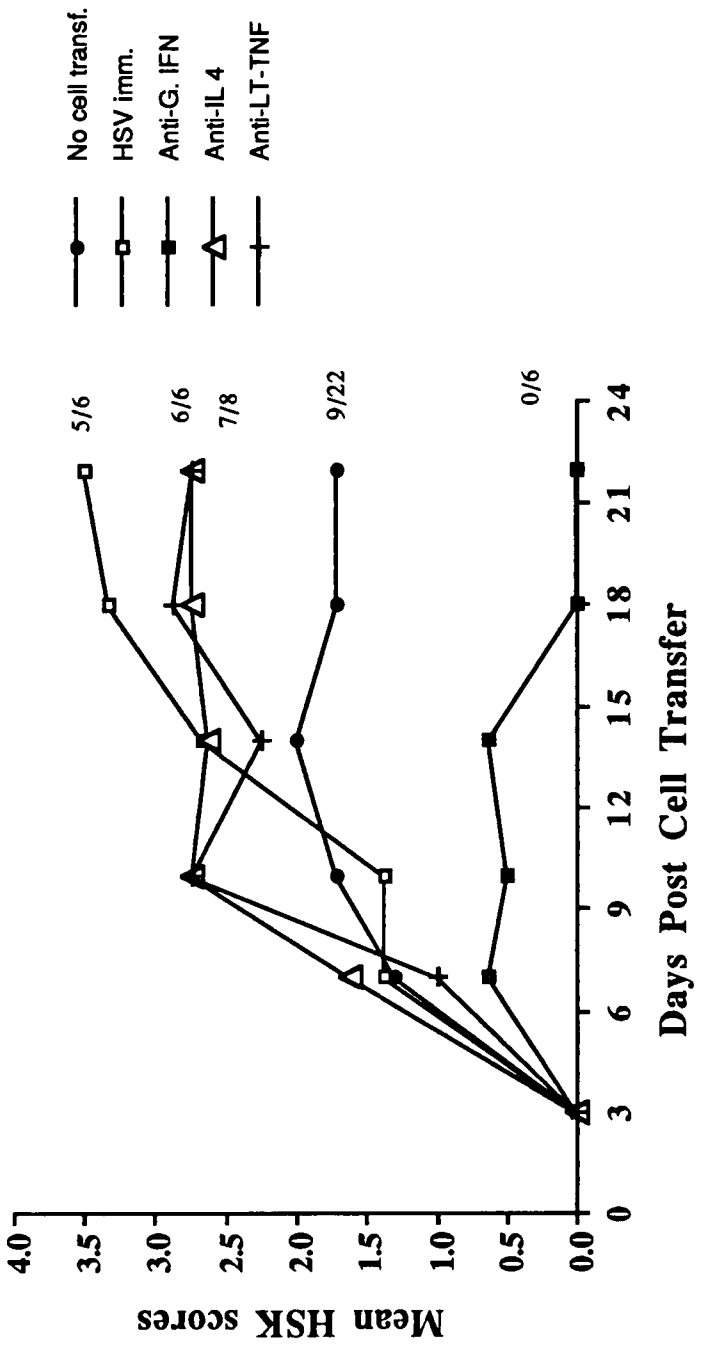
No cell transf.
HSV imm.
Anti-G. IFN
Anti-IL 4
Anti-LT-TNF
5/6
6/6
7/8
9/22
0/6
Mean HSK scores
Days Post Cell Transfer
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
0
3
6
9
12
15
18
21
24

Table 5. 1. The effects of anti-lymphokine antibodies on the development of HSK as studied in immunocompetent Balb/c mice[a].

mAb treatment Groups	Days post infection						
	6	9	12	15	19	22	26
Control mAb	1.3[b]	1.5	1.9	2.8	3.3	3.3	3.4
Anti-CD4	0.5	0.4	0.1	0.7	0.2	0.6	0.6
Anti-CD8	0.9	0.8	1.5	2.3	3.3	4.3	4.0
Anti-IL 2-R	0.6	0.6	1.0	1.3	1.5	1.6	2.3
Anti-G.Interf.	0.3	0.7	1.8	2.2	2.3	2.5	2.4
Anti-IL 4	1.2	1.5	2.0	2.5	2.5	2.2	3.0
Anti-LT-TNF	0.8	1.1	1.9	2.6	3.5	3.9	3.9
Cyclosporin	0.4	0.5	1.8	1.2	1.0	0.8	0.5
Anti-NK	1.0	1.3	1.6	1.1	1.3	0.8	0.7

[a] Control mAb (HB151), anti-CD4 (GK 1.5), anti-CD8 (2.43), anti-IL 2-R (PC 61), and anti-G. Interferon (R 46A2) were all rat IgG and grown as described previously. Anti-IL 4 (11 B 11) was provided by Dr. G. Reynolds at NCI, Frederick, MD.Anti-LT-TNF was provided by N. Ruddle, at Yale Univ.New Haven, CT. Anti-NK (anti-asialo-GM) was purchased from Wako Chemicals USA Inc. Richmond, VA. Control mAb, anti-CD4, Anti-CD8, and Anti-IL-2 R were administered to mice at .5mg/mouse/ injection at days -2, 0, +3, +7, +10 and +17. Anti-G. Interferon (1mg/mouse, Rosenberg et. al., 1990, J. Immunol. 144:4648), Anti-IL 4 (1mg/mouse), and Anti-LT-TNF (300ug/mouse) were injected at +7 day. Anti-NK injection was done once at -2 day at the dose of 50ul/mouse. Cyclosporine was injected (sc) at alternate days at 50mg/kg/day dose starting -2 day. All other agents were injected ip.

[b] The average HSK scores were presented. Corneal challenges were done with RE-HSV ($1x10^6$ $TCID_{50}$) as described previously.

The conclusion of this experiment is that cyclosporin, anti-NK, anti-CD4, anti-IL 2 R, and anti-G. Interferon treatments seem to reduce the HSK in immunocompetent mice. The effects of anti-G. interferon and anti-IL 2 R antibodies do not appear to be as great as the others (cyclo.anti-NK and anti-CD4) probably because of the low dose of the antibodies used in these groups. The dosage of anti-CD4 and anti-NK and cyclosporin are well studied in many models however, the dosage of anti-G. Interferon and anti-IL 2 R are less well defined (the references were given in Fig.5.1 legend). Posible approaches to determine the effects of the treatment with these antibodies could be the demonstration of the direct effect on anti-herpetic immune responses (on DTH, on antibody etc.)and on the development of HSK. Furthermore, after anti-IL-2 R antibody treatment possible reductions in the draining lymph node cells ($CD8^+$ T cells vs $CD4^+$ T cell), should be determined with flow cytometry.

VITA

Mehmet Ziya DOYMAZ was born in a small farming village in the Western Anatolia at an undefined date in the early sixties. After completing his high school at Bursa Imam Hatip Lisesi, which is a formal training school for mullahs, he served for a short while as a religious leader in another small village in central Anatolia. Deciding to study more about this world than the other one, Mehmet entered Ankara Universitesi, Veteriner Fakultesi and graduated in 1983. Following graduation he worked for a short while as a practicing veterinarian in Fethiye, a beautiful resort town nearby the Mediterranean Sea. In 1984, he came to the USA for graduate training on a scolarship from the Turkish Ministry of Education and attended The University of Tennesse, Knoxville, where he received both the M. Sc. and Ph. D. degrees. If the Turkish military allows he plans to pursue post doctoral training at the Mount Sinai School of Medicine in New York before returning to his native land.