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THE ROLE OF CD4+ T CELLS AND THEIR EFFECTOR CYTOKINES IN THE IMMUNOPATHOLOGY OF HERPETIC STROMAL KERATITIS

A dissertation

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Degree

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To my father

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ABSTRACT

Herpetic stromal keratitis (HSK) is an inflammatory reaction of the corneal stroma and considered to represent an immunopathologic response to herpes simplex virus (HSV) infection. In murine models, CD4+ T cells act as an essential participants in manifestation of HSK. The contribution of Th1 (type 1) and Th2 (type 2) cytokines in the disease process was studied in immunocompetent and T cell reconstituted SCID mouse models. When *in vivo* expression of cytokine mRNAs was analyzed and quantified by polymerase chain reaction in the immunocompetent animals, type 1 cytokine mRNAs, particularly IFN–γ, was expressed in the cornea prior to and during the peak clinical lesions. In draining lymph node (DLN) cells of the same animals, except IL-10, both type 1 and type 2 cytokines were expressed throughout the course of the disease. Similarly, in naive or HSV immune CD4+ T cell reconstituted SCID mice, spontaneous expression of high levels of IFN–γ mRNA was evident in the inflammatory cornea and the donor cell cytokine profile did not reflect the cytokine pattern in the diseased cornea. *In vitro* stimulation of reconstituted SCID mice corneal inflammatory cells with HSV resulted in selective secretion of IFN–γ in the culture supernatant, although multiple non secreted cytokine mRNAs could be induced after stimulation. In addition, the inflammatory corneal T cells isolated from the CD4+ T cell reconstituted SCID mice were partially anergic to HSV specific *in vitro* proliferative response. However, IL-4 producing Th2 cells proliferating in response to HSV specific stimulation were demonstrable in the reconstituted SCID mice splenic cells. On the other hand, when some immunocompetent animals with mild disease begin to recover, high levels of type 2 cytokine mRNAs, particularly IL-10, were coexpressed along with IFN–γ in the inflammatory cornea and in the DLN. In addition, cytokine induction was higher at the local site as evident from *in vitro* cytokine secretion from few inflammatory corneal cells, as well as temporal comparison of cytokine mRNA transcription frequency between the corneal and the DLN cells. In HSV infected SCID and nude mouse models, adoptive transfer of I-E^d reactive CD4+ T cells resulted in HSK lesions. The autoreactive T cells were also present in HSV infected immunocompetent and reconstituted SCID mouse models. However, productive

progeny viral infection in the cornea is necessary in the pathogenesis of HSK. Corneal infection with UV inactivated or replication defective HSV mutants did not induce the disease, even in the presence of adequate frequency of HSV reactive CD4+ T cells. Taken collectively, the results indicate that following productive viral infection, multiple cytokine producing CD4+ T cells enter the cornea but selective activation of Th1 cells occur within the corneal milieu and are involved in inducing HSK. The selective activation or recruitment of IL-10 producers in some immunocompetent animals with disease remission suggests that IL-10 may be involved in the disease resolution. The autoreactive T cell responses seen in HSV infected animals, and reconstitution of disease with autoreactive T cells indicate possible involvement of self determinant in activation of CD4+ T cells during the chronic inflammatory process of HSK.

TABLE OF CONTENTS

SECTION		PAGE
	PART I	
	LITERATURE REVIEW	
1.	INTRODUCTION	2
2.	T CELL MEDIATED IMMUNOPATHOLOGY IN INFECTIOUS DISEASES	3
	2.1. Immunopathology of viral infections	3
	2.1.1. Immunopathology of viral infections mediated by CD8+ T cells	4
	2.1.2. Immunopathology of viral infections mediated by CD4+ T cells	6
	2.2. Immunopathology of bacterial infections	9
	2.3. Immunopathology of parasitic infections	11
3.	ROLE OF Th1 AND Th2 CYTOKINES IN IMMUNOPATHOLOGICAL DISORDERS	14
4.	IMMUNOPATHOLOGICAL CONDITIONS IN HSV INFECTION	22
	4.1. Herpetic stromal keratitis	24
	4.1.1. Murine models of herpetic stromal keratitis	24
	4.1.2 Evidence of CD4+ T cell mediated immunopathology in HSK	25
	4.1.3 Proposed mechanisms of CD4+ T cells in the pathogenesis of HSK	26
5.	RATIONALE FOR THE CURRENT STUDY ON THE HSK PATHOGENESIS	29
6.	SPECIFIC AIMS	32
	PART II	
	DEVELOPMENT AND STANDARDIZATION OF QUANTITATIVE POLYMERASE CHAIN REACTION FOR QUANTIFICATION OF MURINE CYTOKINE mRNA PRESENT IN SMALL NUMBER OF CELLS	
1.	ABSTRACT	34
2.	INTRODUCTION	35
3.	MATERIALS AND METHODS	37
	3.1. Cell culture and mRNA preparation	37
	3.2. Construction of the internal standard for quantitative PCR	37

3.3. Reverse transcription and calculation of cDNA synthesis efficiency 37
3.4. Quantitative polymerase chain reaction 37
3.5. Demonstration of primer efficiency in the amplification rate 40

4. RESULTS
4.1. Demonstration of the efficiency of primers in a PCR reaction 41
4.2. T cell cytokine quantitation using SYNTA-M RNA as a standard 41
4.3. Quantitation of T cell cytokine mRNAs by competitive PCR using UT-M as a competitor 45
4.4. Amplification rate of the target and the UT-M at equimole ratio 48
4.5. Expression patterns of T cell cytokines 48

5. DISCUSSION 50

PART III

QUANTITATIVE MEASUREMENT OF *IN VIVO* EXPRESSION OF Th1 (TYPE I) AND Th2 (TYPE II) CYTOKINE mRNA EXPRESSION DURING THE COURSE OF HSK IN EUTHYMIC BALB/c MICE

1. ABSTRACT 54

2. INTRODUCTION 55

3. MATERIALS AND METHODS
3.1. Mice 56
3.2. Corneal infection and clinical observation 56
3.3. Isolation of corneal specimens for RNA preparation 56
3.4. RNA preparation 57
3.5. Reverse transcription 57
3.6. Polymerase chain reaction (PCR) 57
3.7. Qualitative PCR analysis of the cytokine mRNA expression 58
3.8. Primers for the PCR 58
3.9. Internal starndard for quantitative PCR 58
3.10. Quantitative PCR 58

4. RESULTS
4.1. Course of HSK in immunocompetent BALB/c mice 62
4.2. Cytokine mRNA expression in corneas during different clinical phases of HSK 62
4.3. Expression of cytokine mRNAs in the DLN 64
4.4. Change in the expression levels of cytokine mRNA in the cornea and the DLN during the course of HSK 67

5. DISCUSSION 69

PART IV

STUDIES ON THE INDUCTION AND EFFECTOR FUNCTIONS OF HSV-1 RE IMMUNE AND NAIVE CD4+ T CELLS IN THE RECONSTITUTED SCID MOUSE MODELS OF HSK

1. ABSTRACT 74

2. INTRODUCTION 75

3. MATERIALS AND METHODS 77
   - 3.1. Mice 77
   - 3.2. Corneal infection and clinical observation 77
   - 3.3. Donor cell preparation and adoptive transfer 77
   - 3.4. Isolation of corneal specimens for RNA and cell isolation 78
   - 3.5. Culturing corneal cellular infiltrates and preparation of fresh corneal cells from the reconstituted recipients 78
   - 3.6. Lymphocyte proliferation 79
   - 3.7. Stimulation of corneal cells for cytokine induction 79
   - 3.8. Measurement of cytokine secretion in the culture supernatant 80
   - 3.9. Flow cytometry 80
   - 3.10. RNA preparation and quantitative polymerase chain reaction 80

4. RESULTS 83
   - 4.1. Early manifestations of HSK lesions in the immune CD4+ T lymphocyte reconstituted SCID mice 83
   - 4.2. Response of the lymph node and splenic naive CD4+ T cells in the manifestation of HSK 83
   - 4.3. Spontaneous expression of IFN-γ mRNA in the inflammatory cornea 87
   - 4.4. *In vitro* secretion of IFN-γ but not IL-2, IL-4, and IL-10 cytokines from the corneal cellular infiltrates in response to HSV specific stimulation 92
   - 4.5. Corneal T cells that secreted IFN-γ were partially anergic to HSV specific proliferative response but not to mitogen 97
   - 4.6. Self reactivity of the donor cell population in the reconstituted SCID spleen 97
   - 4.7. Differential cytokine response in the recipient splenic cells 99

5. DISCUSSION 107

PART V

ROLE OF CD4+ T CELLS REACTIVE TOWARDS NON-HSV DERIVED DETERMINANTS IN THE PATHOGENESIS OF HSK

1. ABSTRACT 115

2. INTRODUCTION 116

3. MATERIALS AND METHODS 117
   - 3.1. Generation and maintanence of autoreactive T cell line 117
   - 3.2. Antibodies 117
   - 3.3. Lymphocyte proliferation assay 118
   - 3.4. Mice 118
   - 3.5. Corneal infection and clinical observation 118
   - 3.6. Reverse transcription and polymerase chain reaction 119

4. RESULTS 120
   - 4.1. General remarks and characterization of RB 408/CD4 120
   - 4.2. Cytokine pattern of RB408/CD4 120
   - 4.3. Adoptive transfer of RB 408/CD4 into HSV infected nude and SCID mice result in HSK lesions 125
   - 4.4. Spontaneous cytokine mRNA expression in non inflammatory and inflammatory cornea of RB 408/CD4 reconstituted mice 129

5. DISCUSSION 132

PART VI

THE ROLE OF HSV IN POTENTIATING THE IMMUNOPATHOLOGY OF HSK

1. ABSTRACT 136

2. INTRODUCTION 137

3. MATERIAL AND METHODS 139
   - 3.1. Mice 139
   - 3.2. Viruses 139
   - 3.3. Corneal infection and clinical observation 140
   - 3.4. Donor cell preparation and adoptive transfer 140

4. RESULTS 141
   - 4.1. UV inactivated HSV fail to produce HSK in BALB/c mice 141

4.2. Response of the replication defective mutants in the develpoment of HSK in the euthymic BALB/c mice 141
4.3. Response of the mutant viruses following topical corneal infection in the SCID mouse reconstituted with wild type HSV-1 RE or HSV-1 KOS immune CD4+ T cells 143

5. DISCUSSION 148

PART VII

CONCLUSIONS AND FUTURE PERSPECTIVES 152

LIST OF REFERENCE 160

VITA 183

LIST OF TABLES

TABLE PAGE

PART II

1. Expression levels of IL-2, IL-4, IL-5, IFN-γ and β-actin mRNAs in total cellular RNA of activated murine T cells 44
2. Proportion of IL-2, IL-4, IL-5, and IFN-γ mRNAs in relation to β-actin mRNA in Balb/c and C57/BL T cells 46

PART III

1. The sense and antisense primers of murine cytokine mRNAs 59
2. Expression levels of cytokine mRNAs in the cornea during the course of HSK 63
3. Expression levels of cytokine mRNAs in the draining lymph nodes during the course of HSK 65
4. Expression levels of cytokine mRNAs in the draining lymph nodes during the course of HSK 66

PART IV

1. Summary of immune splenic and lymph node CD4+ T cell transfer in reconstituted SCID mice 85
2. Summary of naive splenic and lymph node CD4+ T cell reconstitution in SCID mice cornea 90
3. Expression levels of cytokine mRNA in immune CD4+T cell reconstituted SCID mice 93
4. Expression levels of cytokine mRNAs in HSV immune CD4+ splenic cells used for reconstitution 94
5. *In vitro* cytokine secretion of fresh and cultured inflammatory corneal cellular infiltrates of reconstituted SCID mice 95
6. Self reactive splenic T cells in HSV-1 RE infected reconstituted SCID mice 101

PART V

1. Manifestation of HSK in RB 408/CD4 reconstituted Nude and SCID mice 128

PART VI

1. Summary of HSK manifestation in replication defective andUV inactivated virus infected reconstitutedSCID mice 140
2. Summary of HSK manifestation in replication defective and UV inactivated virus infected SCID mice reconstituted with high cell numbers 142

LIST OF FIGURES

FIGURE PAGE

PART II

1. Design of UT-M 38
2. Demonstration of primer efficiency in the amplification rate 42
3. Quantitative plots using synthetic internal RNA standard 43
4. Representative gel and graph of the competitive PCR using UT-M as a competitor 47
5. Demonstration of identical amplification rate of both target and UT-M at equimole ratio 49

PART III

1. Quantification of cytokine mRNAs by competitivePCR 61
2. Change in the temporal frequency of cytokine mRNAs transcription during the course of HSK 68

PART IV

1. Experimental design of reconstituted SCID mouse models of HSK 82
2. Dynamics of HSK manifestation in immune CD4+ T cell reconstituted SCID mouse models and in euthymic BALB/c mice 84
3. Manifestation of HSK in the naive splenic and lymph node CD4+ T cell recipients and percentages of animals manifested the lesions 86
4. Flow cytometric analysis of the naive CD4+ donor T cells 88
5. Histological sections of immune and naive CD4+ T cell reconstituted and non reconstituited SCID mice cornea 91
6. Qualitative and semiquantitative RT-PCR analysis of *in vitro* stimulated corneal inflammatory cells at the end of *in vitro* stimulation 96
7. Lack of HSV specific proliferative response in the HSK corneal cells 98
8. A representative self reactive and HSV specific responses in the reconstituted SCID mouse spleen cells 102
9. Self reactive responses in HSV immune T cells of BALB/c mice 103
10. Cytokine secretion pattern of self and HSV reactive T cells in the reconstituted SCID splenic cells 104

PART V

1. Autoreactive responses of RB 408/CD4 121
2. IE-^{d} reactive response of RB 408/CD4 122
3. Cytokine profile of RB 408/CD4 123
4. *In vitro* growth response of RB 408/CD4 to rIL-2 and rIL-4 124
5. *In vitro* cytokine secretion of RB 408/CD4 in response to syngeneic stimulators 126
6. HSK manifestation in RB 408/CD4 reconstituted nude mice 127

7. Histology of HSK lesions in RB 408/CD4 reconstituted nude mouse 130
8. Cytokine profile in RB 408/CD4 reconstituted nude mice inflammatory and non inflammatory corneas 131

PART VI

1. HSK and DTH manifestation in UV inactivated and live HSV infected BALB/c mice 142
2. HSK manifestation in BALB/c mice infected with replication defective HSV mutants 144

PART I

Literature Review

1. INTRODUCTION

The effector arms of the immune system, both cell mediated immunity (CMI) and humoral response, usually play a protective role in a host against foreign agents. However, when the normally protective components of the immune system become dysfunctional, degenerative host tissue damage is encountered in certain locations. The pathological manifestations caused by exuberant immune responses is termed as immunopathology (IP).

IP may vary depending on the nature of the manifestations and range from mild allergic reactions to serious illness, such as various organ specific disorders. In general, the term IP is used in conjunction with a known etiological agent or the specific organ afflicted. If the effector arms of the immune system are involved in spontaneous manifestations of certain organ specific disorders, the term auto immune disease is commonly employed (Kumar, et.al., 1989). However, it is now evident that both the autoimmune diseases and organ specific disorders caused by immune response to a known etiological agent behave in a similar manner. Although IP may be induced by non infectious agents, such as allergens, infectious agents have been suspected to initiate one form of organ specific disorder or another. In fact, though direct evidence in clinical situations are lacking, infectious agents are suspected to play a role in inducing autoimmune diseases (Theophilopoulos, 1995)

In many immunopathological conditions, both humoral and CMI responses are involved in the resultant organ specific dysfunction. However, the current discussion is confined manily to IP of certain infectious diseases mediated by CMI. In the following sections, some selective immunopathological conditions involving infectious agents are reviewed which form a basis for understanding the CD4+ T cell mediated IP of herpetic stromal keratitis (HSK) in the murine models induced by herpes simplex virus (HSV).

2. T CELL MEDIATED IMMUNOPATHOLOGY IN INFECTIOUS DISEASES

CMI against most infectious agents are generally successful in limiting the spread of or clearing the infection. Such CMI not only play a prinicipal role in recovery from primary infection, but also offer a protective immunity against secondary infection (Nash et.al., 1985 and Rouse, 1988). However, many pathogens establish persistent infection in the host at the initial site of infection. Whether persistent infection occurs as a result of incomplete generation of protective immunity or by evasion of immune system by the pathogen is arguable (Banks and Rouse, 1993). However, under normal clinical situations in humans, various organ specific pathological conditions occur as a sequel to the persistent infection.

Much of our understanding of IP of human diseases caused by various infectious agents, such as viruses, bacteria, and parasites comes from animal models. However, animal studies on the IP of human diseases have a serious limitation. Unlike the natural course of IP in humans that is manifested as a result of progressive immune response due to chronic infection, experimental immunopathological conditions in animal models are usually studied following acute infection. Nevertheless, animal models have helped us to understand the basic mechanisms of various immunopathological conditions. Accordingly, in animal models of T cell mediated IP caused by infectious agents, the pathology is not manifested in immunocompromised animals by infectious agents alone. However, in the presence of functional T cells, the disease is readily manifested and allows a model system to dissect the sequence of events behind immunopathological conditions mediated by infectious agents.

2. 1. Immunopathology of viral infections

The consequences of viral infections in a host vary from immediate virus induced inflammatory response to IP and the resultant tissue damage of the various organs. In immunocompetent host, the direct tissue damage during the acute phase of viral infections are generally self limiting and the virus is eliminated from the host by the induced immunity (Sissons and Borysiewicz, 1985). However, in certain viral infections, the induced CMI against the viral antigen result in immunopathological conditions in various tissues. Although in persistent viral IP the CMI is directed against

the viral antigens, in some occasions the cross reactive viral antigens to the host self antigens (molecular mimicry) (Oldstone, 1987) or release of sequestered self antigens that are not normally encountered by the immune system by inital viral infection is also considered to play a role. For instance, various viral infections have been implicated in the etiology of multiple sclerosis, and insulin dependent diabetes mellitus in humans (Gamble, 1980). Accordingly, pathogens such as viruses may provide the initial stimulus through increased presentation of sub-dominant determinants, either by molecular mimicry (Gammon et.al., 1991) or exposure of cryptic determinants for antigen presentation. Furthermore, once a response against cryptic or sub-dominant determinants has been initiated, there may be second waves of responses against additional cryptic determinants exposed from other molecules through the process of determinant spreading (Lehmann et.al., 1993 and Sercarz et.al., 1993). In the absence of immune response, as in immunocompromised host, although certain viral infections might be fatal, in most situations the viral infection is confined by inate immunity without any organ specific IP (Schattner and Rager-Zisman, 1990). However, in the immunocompetent host, the CMI elicited by the pathogen inflicts host tissue damage in certain locations.

The nature of the T lymphocyte subsets, namely CD4+ or CD8+ cells, involved in IP appear to vary depending on the immunopathological conditions. Some of the virus induced IP in which either the CD4+ or CD8+ T cells have been well studied are briefly reviewed below.

2. 1. 1. Immunopathology of viral infections mediated by CD8+ T cells:

One of the well studied animal models in which the CD8+ T cells have been implicated in IP is lymphocytic choriomeningitis virus (LCMV) infection of mice. Although human infections of the LCMV and the resultant pathological conditions is not present, nevertheless, the results obtained with the IP of LCMV infection form a prototype of other viral infections. LCMV exhibits a pan tropism and infects various tissues, including T lymphocytes. In immunocompromised animals, the virus is disseminated in various organs leading to persistent infections without any organ specific disorders (Buchmeier et.al., 1990). On the other hand, in immunocompetent animals fatal inflammation of choroid plexus and leptomeninges occurs following intracranial inoculation of the virus (Allan et.al., 1987). In immunocompromised

animals, the pathology associated with initial acute viral infection in the CNS (central nervous system) is confined to the focal area of viral infection and resolves without any serious outcome. However, in the presence of immune CD8+ cytotoxic T lymphocyte (CTL), extensive tissue damage ensues. Accordingly, both in persistently infected animals or during acute infection, intracranial inoculation of either cloned or polyclonal LCMV immune CD8+ CTL results in fatal inflammatory response of the CNS (Beazinger et.al., 1986 and Dixon et.al., 1987). Furthermore, in persistently infected immunocompromised animals in which disseminated viral infection is seen, intravenous transfer of immune CD8+ CTL also results in inflammation of the lung, in addition to CNS inflammatory response (Byrne and Oldstone 1986). Although the presence of viral antigen in the local site of infection has been identified by the immunoflurosence technique, the virus itself appears to play a minimal role in tissue damage (Allan et.al., 1987). Thus the disease appears to result in response to host CD8+ CTL. Accordingly, in the inflammatory reactions, participation of CD4+ T cells has been found to be minimal and virtual absence of CD4+ T cells was observed in the inflammatory reaction (Doherty et.al.,1990). However, in the absence of CD8+ T cells, CD4+ T cells appear to play a role in the inflammatory process. In this regard, Muller and colleagues (1992) have observed the IP in β_2M- deficient mice that are genetically deficient in CD8+ T cells.

Hepatitis B virus (HBV) is an another example of virus induced IP mediated by CD8+CTL cells. *In vitro* infection of hepatocytes with HBV results in non cytolytic viral replication and it is assumed that perhaps the HBV infection *in vivo* is also non cytodicidal for the liver cells (Hollinger et.al., 1990). Thus, much of hepatic tissue injury seen in acute viral infection is thought to be mediated by the components of the host immune system. The presence of CD8+ T cells in diseased liver as well as the presence of HBV capsid antigen specific CD8+ CTL in circulatory lymphocytes initially suggested a role for HBV specific CD8+ CTL in the disease (Mondelli et.al.,1990). More definitive evidence that virus specific CD8+ T cells play a role came from transgenic mouse experiments.

Transgenic mice expressing the hepatitis B surface Ag (HBsAg) in liver cells were developed (Moriyama et.al.,1990). In this animal model, adoptive transfer of either HBsAg specific CD8+ CTL clone or polyclonal immune CD8+ CTL resulted in extensive necrosis of hepatocytes, suggesting the direct role for CD8+ T cells in hepatic

tissue destruction. In addition, recent experiments on this transgenic model indicated that the tissue destruction mediated by adoptively transferred HBsAg specific CD8+ CTL is through the direct antigen dependent activation of the transferred CD8+ T cells *in vivo*. Accordingly, when HBsAg specific cloned CD8+CTL were transferred to HBsAg transgenic animals lacking in the appropriate MHC I restricting elements, hepatic tissue destruction was not evident, even though the transferred cells could readily be detectable in the liver sinusoids (Ando et. Al., 1993 and 1994). These results clearly indicated that MHC I restricted antigen specific activation of the transferred CD8+ CTL *in vivo* resulted in the hepatic tissue destruction. Although, CD8+ T cells have been implicated in the HBV induced IP, the role of CD4+ T cells have not been well studied. It is yet to be documented in HBsAg transgenic mice whether transfer of HBsAg specific CD4+ T cells could also result in liver injury.

Mycocarditis in coxsackie viral infection of mice is an another animal model of viral IP in which T cells have been implicated in the pathogenesis (Woodruff and Woodruff 1974). As in various models of virus induced immunopathological lesions, in immunocompromised mice, the viral infection alone does not induce myocarditis. However, adoptive transfer of immune T cells result in manifestation of cardiac tissue injury (Blay et.al., 1989). The nature of T cell subsets participating in the IP appear to vary between the animal models studied. While in DBA mice CD4+ T cells have been implicated in the disease process, in BALB/c mice both CD4+ and CD8+ T cells are thought to play a role (Van Honten and Huber, 1984). In addition, to viral antigen reactive T cells in the virus infected mice, T cells reactive to cardiocyte were also noted (Estrin and Huber, 1984 and Gutrie et.al., 1984). Although reports on T cell antigen specificity at the inflammatory myocardium is lacking, adoptive transfer of both virus and cardiocyte reactive CD8+ T cells have been demonstrated to result in myocarditis (Gutrie et.al., 1984). Thus, IP of coxsackie viral infection is, perhaps, a direct animal model for the virus induced auto immune pathology.

2. 1. 2. Immunopathology of viral infections mediated by CD4+ T cells

As reviewed above, in certain viral immunopathological conditions CD8+ CTL appear to be predominant participants in the efferent phase of the immunopathological lesions. However, in certain viral infections CD4+ T cells are also involved in IP. In this section three viral infections that have been well characterized are briefly reviewed.

Because of suspected etiology of viral infection in the development of multiple sclerosis in humans, murine models of Theiler's murine encephalomyelitis virus (TMEV) infection have been considered as an animal model to study the IP of CNS disorder (Miller and Karpus, 1994). Perhaps the direct evidence to link the viral infection in the natural course of demyelinating disease could be found in the natural infection of Marek's viral infection of chickens (Sell, 1987). TMEV is a natural pathogen of mice and, as in LCMV IP, intracerebral infection of infectious TMEV results in life long persistent infection of CNS white matter. However, as a result of persistent infection, in the immunocompetent mice chronic CNS pathology associated with primary demyelination accompanied by mononuclear cellular infiltration is seen. In susceptible mouse strains, intracerebral infection with TMEV results in peripheral viremia which is resolved with concomitant development of neutralizing antibodies. Although the virus specific neutralizing antibodies control the viremia, persistence of virus in the CNS is seen. As a consequence, the CMI response induced to initial viral infection causes a debilitating demyelination.

Earlier studies have implicated a role for CD4+ T cells in the demyelination process. Accordingly, *in vivo* depletion of CD4+ cells during early phase of viral infection resulted in extensive disseminated viral infection leading to death of the animal. On the other hand, if depletion of CD4+ T cells was delayed, the severity of demyelination was reduced and the disease eventually resolved (Welsh et.al., 1987). Although some studies have implicated CD4+ T cells in the demyelination process (Clatch et.al., 1986), some schools of thought also implicate CD8+ CTL in the CNS disorder. Accordingly, as in CD4+ T cell depletion studies, depletion of CD8+ T cells also reduced the severity of demyelination (Rodriguez and Sriram, 1988). It is possible that both subsets may be involved in IP. Initially, it was susepected that the target antigen recognized by T cells infiltrating the CNS may be myelin derived peptides (Clatch et.al., 1985 and 1986). However, the notion was strongly questioned when TMEV immune T cells failed to produce demyelination in naive syngeneic recipients following adoptive transfer (Rodriguez et.al., 1986). The presence of virus specific delayed type hypersensitivity (DTH) reaction as well as T cell proliferation in affected mice suggest that at least some of the T cell responses in CNS may be associated with virus specific activation (Clatch et.al., 1986). In fact, in SJL/J mice the viral nucleocapsid protein (VP2) derived pepide amino acids 74-86 have been implicated as

target antigen for the CD4+ T cells (Geretz et.al., 1994). Thus, the virus that persists in the CNS may serve as a reservoir for activation of virus specific CD4+ T cells of DTH phenotype in the brain. In this regard, induction of split tolerance to TMEV specific CD4+ DTH cells abrogated the IP (Peterson, et.al., 1993).

Borna disease virus (BDV) infection is an another well studied animal model of virus induced CNS disease in which host CD4+ T cells act as culprits in the pathogenesis (Richt et.al., 1989). In nature, BDV infections are commonly seen in horses and sheep and cause encephalopathy resulting in paralysis and death (Ludwig et.al., 1988 and Richt et.al., 1992). In experimental conditions, BDV exhibits a wide range of host tropism infecting chickens as well as non human primates. In Lewis rat model, the pathogenesis of BDV associated encepathalopathy was ascribed to CD4+ T cells. Neurological lesions and resultant CNS inflammatory response do not occur in virus infected immunocompromised or athymic rats infected with BDV (Herzog et.al., 1984 and Stitz et.al., 1989). On the other hand, adoptive transfer of CD4+ T cells from acutely infected animals result in CNS disorder (Richt et.al., 1990). Histopathological findings were similar to that of the natural course of infection seen in immunocompetent animals and offered compelling evidence that CD4+ T cells are involved in pathogenesis. However, recent studies have indicated that the pathogenic CD4+ T cells can also offer protection from the BDV viral spread within the CNS. Accordingly, passive transfer of BDV specific CD4+ T cell clones before viral infection indeed demonstrated virostatic effect, and abrogated the IP. On the other hand, when transfer of CD4+ T cells was carried out after viral infection, inflammatory tissue damage of the white matter was seen (Richt et.al., 1994).

Respiratory Syncytial Virus (RSV) infection induced pulmonary pathology was also considered to be mediated by host CD4+ T cells. However, immunopathological manifestations of the disease appears to be markedly different from that of various virus induced immunopathological conditions discussed above. RSV associated pulmonary disease was first noticed in humans who have been previously vaccinated with formalin inactivated RSV (FI-RSV) vaccine preparations (Kim et.al., 1969). Although FI-RSV vaccination induced a humoral response, exposure of the vaccinated individuals with subsequent natural RSV infection resulted in damage to the lung. Animal studies with BALB/c and cotton rats have indicated a role of host T cells in the pathological process. Primary infection of the lung with RSV develop pulmonary lymphocytosis with

concomittant development of virus specific CD8+ CTL response. However, depletion of T cells (CD4+ and CD8+) reduces or abolishes the disease while enhancing the viral replication (Graham et.al., 1991). Adoptive transfer of CD8+ CTL lines or clones in such mice, although eliminated viral infection *in vivo*, caused hemorrhagic, neutrophilic, and at times fatal pneumonia (Cannon et.al., 1988). However, the disease more closely resembling as that of natural human disease was demonstrable in animals that have been previously immunized with FI-RSV or immunized with sub-unit vaccine preparations (Murphy et.al., 1990, Vaux-Peretz et.al., 1990 and Wathen et.al., 1991). In this model, the disease is primarily mediated by host CD4+ T cells. However, unlike primary infection with live RSV that result in immediate immunopathological lesions (Graham et al., 1991), FI-RSV immunization does not induce the lung pathology, although CD4+ T cells were efficiently stimulated by the inactivated viral preparations (Brideau and Wathen, 1992). On the other hand, pulmonary pathology is seen only after secondary challenge with live RSV. In such reinfection associated lung disease, depletion of CD4+ T cells abrogated pulmonary lesions (Connors et.al., 1992). However, unlike primary live RSV infection induced immunopathological responses that was dominated predominantly by neutrophil infiltration (Cannon et.al., 1988), in the lung lesions of mice that had been previously immunized with FI-RSV, a predominance of eosinophil infiltration was observed. Thus, the nature of the antigen used during initial priming tends to influence the type of inflammatory cellular infiltrates as well as host T lymphocyte participation in the pulmonary IP of RSV infection (Graham et.al., 1993).

2. 2. Immunopathology of bacterial infections

IP is a not an infrequent consequence of viral infections but is also encountered in bacterial infections, as well. Like viruses, bacterial infections tend to cause initial pathogen induced inflammatory responses. Although with the advent of modern antibiotics many bacterial infections can be therapeutically controlled, in certain bacterial infections, organ specific disorders caused by induced immunity occur. In the following section, a brief review of bacterial immunopathological conditions that are relevant to the current study is discussed.

Listeria monocytogenes, an intracellular Gram-positive bacterium, induces a strong CMI responses *in vivo*. The systematic study of experimental infections in

murine models has provided insight into the nature and quality of T cell-dependent acquired immunity in the development of organ specific IP. In murine listeriosis, the liver bears a heavy bacterial burden caused multiplication of the bacterium within hepatocytes. However, following primary infection, the bacteria are cleared from visceral organs and resolution of primary listeriosis eventually occurs without any deleterious effect on the host (Hahn and Kaufmann, 1981). Although in the immunodeficient hosts (nude and SCID mice) the innate immunity could offer some degree of protection during primary infection, the resolution of primary listeriosis and the ensuing state of long lasting protection from primary as well as secondary infection do not occur (Lane and Unanue, 1972, and Hahn et.al., 1981). Studies conducted on euthymic mice indicate that these functions are exclusively confined to CD4+ T cell mediated acquired immunity (Mielke et.al., 1988). However, recently convincing evidence was also presented showing the protective role of Thy-1+, CD-, CD8- T cells in primary as well as secondary listeriosis (Dunn and North, 1991 and Hiramatsu et.al., 1992). Although *Listeria* specific CD8+ CTL were superior in early control of bacterial spread in high challenge inocula, its role in lasting immunity was found to be minimal (Bishop and Hinrichs, 1987 and Mielke et.al., 1988).

Even though in immunocompetent animals primary listeriosis is resolved, during reinfection accelerated granuloma formation with accompanying DTH response to listerial antigen is seen. The granuloma formation occurs as a result of acquired immunity to *Listeria*. Depletion of CD4+ T cells during reinfection abrogates the granuloma formation, and CD8+ T cell depletion has no effect (Mielke et.al., 1988). Thus, the granuloma formation during reinfection is considered to be mediated by classical CD4+ DTH phenotype cells. In consequence to granuloma formation in the liver and also in spleen, the resulting inflammatory response causes severe compromise of the organ function in the host with often fatal outcome.

Other bacterial infections in which the induced immunity in host that results in adverse immunopathological consequences are mycobacterial infections. *Mycobacterium leprae* infection of humans result in manifestation of leprosy and constitutes one of the oldest disease known. However, leprosy comprises a clinical and immunological spectrum and the type of clinical lesion seen correlates with the type of immunity to M.*leprae* antigens (Bloom et.al., 1992). The leprosy lesions are manifested either as a tuberculoid or lepramatous forms. In tuberculoid form, organized

granulomas with prominant CD4+ T cell infiltrates and strong CMI develop, resulting in killing of most bacilli by macrophages. Although the CMI responses clear the bacterial load at the local site, the infiltrating CD4+ T cells induce a DTH reaction leading to tuberculoid lesion development. On the other hand, lepramatous form is characterized by selective absence of CMI with a predominance of CD8+ T cells in the local lesions (Van Voorhis et.al., 1982 and Modlin et.al., 1983). Although CD8+ T cells are present in the local site, clearance of the bacterium does not occur, and the bacillus multiplies at high levels in macrophages and Schwann cells. During the reactional state of lepramatous lesions to tuberculoid form, the cellular infiltration changes at the local site from CD8+ T cells to CD4+ T cells with augmented CMI response to M. *leprae* antigens (Bjune et.al., 1976, Modlin et.al., 1983 and Cooper et.al., 1989). Although neither the tuberculoid nor the lepramatous lesion is of any cosmetic value to the host, as will be discussed later, leprosy offers an unique clinical settings to understand the consequences of T cell derived cytokines in inducing the IP conditions.

Tuberculosis of lung as a result of Mycobacterium tuberculosis infection was long known to be a IP consequence of host immune response to bacterial antigens (Kaufmann, 1993). Although different species of the M.*tuberculosis* have been implicated in the disease in different species, zoonotic infections are not uncommon in humans. The tuberculoid formation in the lung is associated with host CD4+ T cell immune responses to bacterial antigens. As in tuberculoid lesion development in leprosy, the tuberculous lesions in M. *tuberculosis* are associated with an augmented DTH response of bacterial antigen reactive CD4+ T cells (Hahn and Kaufmann, 1981 and Barnes et.al., 1989).

2. 3. Immunopathology of parasitic infections

In the current context of viral IP perhaps the discussion on parasitic IP might appear irrelevant. However, understanding the basic differences between the parasitic IP will provide a greater insight into the mechanisms of HSK. As it will be dicussed later, controversy exists regarding the effector function of CD4+ T cells in mediating HSK. Furthermore, since the current concept of T cell immunobiology was indeed developed from the findings of parasitic model system, a brief review would be of advantage.

The best studied parasite-induced immunopathological reaction is the schistosome egg granuloma formation. Schistosomiasis is a chronic helminthic disease and the morbidity in schistosome infection is primarily due to fibrosis of the affected tissues as a result of granuloma formation (Boros , 1989). Induced by ova swept from the vasculature where the adult worms reside into tissues such as liver, gut, lungs, and bladder, the initial granuloma are composed primarily of inflammatory cells eventually evolves into a fibrotic lesion causing organ disorder. In murine model, the granuloma formation is greatly reduced by depletion of CD4+ T cells (Mathew and Boros, 1986) and can be restored in athymic nude mice, that normally fail to manifest granuloma formation, by adoptive transfer of purified CD4+ T cells (Cheever et.al., 1989). The granuloma formation was considered to be a DTH response to parasite ova derived antigens (Warren et.al., 1967) because of its CD4+ T cell dependency and DTH reaction in skin to soluble antigens extracted from eggs (Mathew and Boros, 1986). However, currently the granuloma formation is viewed as a more complex immunopathologic entity. In murine models, the peak granuloma formation (7-9 wk post infection) occurs during the time when IgE and circulating eosinophil levels are at their peak (Colley, 1981). Thus, unlike the tuberculoid lesions seen in mycobacterial infection (van Voorhis et.al., 1982 and Barnes, 1989) or the granulomatous lesions seen during reinfection with listeria (Bishop and Hinrichs, 1987 and Mielke et.al., 1988) in which the inflammatory cells are mainly of macrophages and neutophils, in the granulomatous lesion of the schistosomiasis, eosinophils are the predominant inflammatory cellular infiltrates (Boros, 1989), as observed in IP of lung in FI-RSV immune individuals during reinfection (Murphy et.al., 1990, Varex-Peretz et.al., 1990 and Wathen et.al., 1991). Such histological differences of the lesions tend to suggest that perhaps the mechanisms of IP conferred by the CD4+ T cells might be different from that of classical DTH response.

Another parasite induced CD4+ T cell dependent IP is cerebral malaria. This often fatal consequence of *Plasmodium falciparum* infection is considered to result from DTH response of the parasite reactive CD4+ T cells (Sher and Coffman, 1992). Accordingly, in certain strains of mice, histopathological changes similar to those found in human cerebral malaria can be induced by infection with *Plasmodium*, but they are prevented if the mice are depleted of CD4+ T cells (Grau et.al., 1986). However, unlike the granulomatous lesions of schistosomiasis, cerebral malarial inflammatory lesions

contain inflammatory cells reminiscent of DTH respons, even though both pathological conditions are manifested by parasitic infections.

3. ROLE OF Th1 AND Th2 CYTOKINES IN IMMUNOPATHOLOGICAL DISORDERS

In earlier immunological concepts, CD4+ T cells were traditionally viewed as helper cells in aiding B cell immunoglobulin synthesis and promoting DTH function. On the other hand, CD8+ T cells were considered to be cytotoxic T cells and suppressor cells. However, the cytotoxic and suppressor functions are also seen in CD4+ T cells (Schmid, 1988, Yasukawa et.al., 1988 and Tite, 1990). Similarly, CD8+ T cells also exhibit DTH response in certain situations (Ahmed et.al., 1988 and Moskophidis et.al., 1990). Thus, it has become increasingly clear that functional differences between these two T cell subsets are not as dichotomous as it was once considered and, as reviewed in the earlier sections both T cell types can exert their functions in certain immunopathological disorders.

The current views of T cell immunobiology on CD4+ and CD8+ subsets are further refined in accordance with specific effector cytokines secreted by T cells in response to antigen specific stimulation. Based on the cytokine secretion pattern in long term culture, Mosmann and Coffman (1986, and 1989) initially suggested that murine CD4+ T cells can be distinguished into two categories as Th1 and Th2. Accordingly, the Th1 cells secrete IFN–γ, IL-2, and TNF-α, while the Th2 cells secrete IL-4, IL-5, IL-6, or IL-10. Although the original definition "Th" was coined to designate the two polarized responses seen within the traditionally viewed CD4+ T "helper" cells, it is now clear that such a designation could also be extended to CD8+ T cells, as well. Accordingly, CD8+ CTL has also shown recently (Sad et.al., 1995) to secrete dichotomous pattern of cytokines, and TC1 and TC2 nomenclature is proposed for CD8+ CTL that secrete cytokines similar to CD4 + Th1 and Th2 cells, respectively . However, since the cytokine profile is same in both CD4+ and CD8+ T cells, in the following sections Th1 and Th2 nomenclature is used along with CD4+ and CD8+ designation for simplicity. The Th1 or Th2 cells of either CD4+ or CD8+ T cell lineage appear to play a direct role through their effector cytokine release in various immunopathological disorders reviewed earlier. In the current context of Th1 and Th2 classification, it is now generally considered that the Th1 cells are involved in the CMI responses (DTH and CTL) and the Th2 cells are more efficient helper cells for antibody

production. In addition, both Th1 and Th2 cells also negatively regulate each others function. Accordingly, the Th1 cells suppress the effect of Th2 cells, and *vice versa via* their effector molecules (Mossmann and Coffman, 1989)

The significance of polarized Th responses that determine the outcome in an infectious disease could be found in murine models of leishmaniasis. The protozoal infection does not cause an immunopathological response and much of the tissue damage is mediated by activated macrophages in response to multiplication of protozoa (Sher and Coffman, 1992). Nevertheless, murine leishmaniasis offer an excellent model system to study the consequences of polarized Th1 or Th2 cytokine induction that offer resistance or susceptibility to infection. Cutaneous infection of most inbred stains of mice with *Leishmania major* leads to localized lesions that heals spontaneously and confers resistance to reinfection. However, in suscepible strains of mice, such as in the prototype BALB/c, the infection progresses into a disseminated visceral disease that culminates in host mortality. In analysing the cellular basis of this disease pattern, Liew and colleagues (1989) have shown that resistance and susceptiblity to parasite infection is due to dichotomous cytokine secretion pattern of the CD4+ T cells. Clear evidence now exists that resistance and susceptiblity to infection between inbred mouse strains reflect the development of strong CD4+ Th1 and CD4+ Th2 responses, respectively. In susceptible BALB/c mice, CD4+ T cells express high levels of IL-4 mRNA but little IFN–γ mRNA. On the other hand, in the resistant mouse strains, such as C57/BL, higher levels of IFN–γ but no IL-4 mRNA was evident (Heinzel et.al., 1989 and 1991). Similarly, *in vitro* stimulation of the CD4+ T cells from infected animals with *Leishmania* antigens demonstrated the same polarized cytokine responses; BALB/c mice CD4+ T cells secreted higher levels of IL-4, IL-5 and little or no IFN–γ and C57/BL mice CD4+ T cells secreted IFN–γ but not the others (Sadick et.al., 1986, Scott et.al., 1989, Boom et.al., 1990 and Coffman et.al., 1991). Furthermore, the genetic resistance between these two mouse strains can be abrogated by adoptive transfer of *Lesihmania* antigen specific CD4+ Th2 or Th1 clones, respectively. Thus, *Leishmania* specific CD4+ Th1 cells can offer protection to the normally susceptible BALB/c mice, whereas transfer of CD4+ Th2 cells exacerbates the parasitic infection in the resistant mouse strain (Liew et.al., 1985 and 1987).

Studies on the mechanisms by which the CD4+ Th1 and Th2 cells dictate the outcome of resistance and susceptibility to infection have indicated the primary role of IFN–γ and IL-4, respectively in the disease process. The CD4+ Th1 cells mediate DTH response (Cher and Mosmann, 1987) while the CD4+ Th2 cells suppress the DTH of Th1 cells *via* IL-4 or IL-10 (Fiorentino et.al., 1989 and 1991). The presence of strong DTH reactions to leishmanial antigens in resistant mice (Liew et.al., 1989), and transfer of protection to susceptible mice by Th1 cells (Scott et.al., 1988) cumulatively suggest that the CD4+ Th1 cells of DTH phenotype are involved in conferring resistance to infection. Accordingly, neutralization of IFN–γ early on in the infection converted the resistant mice susceptible to infection (Belosevic et.al., 1989 and Scott, 1991). Conversely, neutralization of IL-4 in the susceptible mouse strain cured them from leishmaniasis (Sadick, 1990). However, clearance of protozoa is primarily mediated by macrophages activated in response to IFN–γ secreted by CD4+ Th1 cells in addition to synergistic activation by TNF-α, that is also from Th1 cells (Murray et.al., 1983, Bogdan et.al., 1990, and Liew et.al., 1990). Perhaps the direct evidence of the role of IFN–γ in offering protection from leishmaniasis was demonstrated by Wang and colleagues (1994). Accordingly, the genetically resistant C57/BL mice that was rendered IFN–γ deficient became susceptible to leishmaniasis. Thus the immunosuppression as a result of CD4+ Th2 cell activation was considered to offer a conducive environment for the parasite survival (Sher and Coffman, 1992) in susceptible mice.

In contrast to the protective effect of Th1 cells seen in leishmaniasis, with few exceptions, in many viral and bacterial infection induced immunopathological reaction, the Th1 immune responses are involved in host tissue damage. In both LCMV(Beazinger et.al., 1986, Dixon et.al., 1987 and Byrne and Oldstone, 1986) and HBV(Moriyama et.al., 1990) virus infections in which CD8+ CTL play a role in manifestation of the immunopathological reactions, the tissue damaging inflammatory response was once considered to be as a result of direct cytotoxic function of the CD8+ T cells (Ando et.al, 1993 and 1994). However, two school of thoughts exist with regard to the mechanisms by which the CTL exert the cytolytic functions. While some suggest the cytolytic mechanism to be as a result of granzyme and perforin mediated target cell necrosis (Podack et.al., 1985, Duke et.al., 1988), others suggest that the target cell destruction by CD8+ CTL occur *via* apoptosis (Zychlinsky et.al., 1991, Duke et.al.,

1983, Ostergaard and Clark, 1989, and Henkart, 1985). Nevetheless, CD8+ CTL secrete Th1 cytokines, IFN–γ and TNF-α, and are known to promote both granzyme release and apoptosis (Laster et.al., 1988). Accordingly, in both LCMV and HBV IP, the Th1 cytokines secreted by CD8+ CTL were considered to play a role in inducing the organ specific disorders. For instance, in LCMV infection IFN–γ bioactivity was detected in the cerebrospinal fluid (Frei et.al, 1988) and the expression of IFN–γ and TNF-α mRNA was markedly increased in the brain (Campbell et.al., 1994). In particular, the expression of IFN–γ mRNA (Campbell et.al., 1994) and protein (Frei et.al., 1988) closely overlapped with the progression of IP. Similarly, in recent studies using HBsAg transgenic mouse model, HBsAg specific CTL clones that secrete either IFN–γ or TNF-α was shown to cause the hepatic tissue damage. Although CTL induced apoptosis of hepatic cells was proposed as a mechanism, *in vivo* neutralization of either one of the cytokines during reconstitution of CD8+ CTL minimized the resultant pathology (Ando et.al., 1993 and 1994).

Similarly, in CD4+ T cell mediated immunopathological responses, Th1 cells were incriminated in the tissue damaging inflammatory responses. With the exception of FI-RSV and schistosomiasis, the encephalopathy and the unremitting demyelinating disease of the CNS in TMEV was associated with Th1 response (Miller and Karpus, 1994). Infact, induction of peripheral DTH tolerance to TMEV specific CD4+ DTH phenotype (Th1) cells, the IP of CNS was abrogated or diminished (Peterson et.al., 1993). Similarly, in BDV induced immunopathological responses,the disease promoting cell types were considered to be mediated by Th1 effectors (Richt et.al., 1994).

The direct role of Th1 cells in the immunopathological reaction is evident in the clinical manifestation of leprosy. Unlike the protective effect of Th1 cells seen in leishmaniasis (Liew et.al., 1985 and 1987 and Scott et.al., 1988), in the tuberculoid lesions of leprosy, the Th1 cytokine mRNAs were expressed in higher levels (Yamamura et.al., 1991, Walker et.al., 1992). Furthermore, local injection of IFN–γ in lepramatous lesions in which the bacillus is present, caused the lesions to become tuberculoid form with concomittant clearanace of the bacterium (Nathan, et.al., 1986 and Kaplan and Cohn, 1991) Thus, although the DTH response mediated by CD4+ Th1 cells aids in clearance of the pathogen from the local site, the cost appears to be tuberculoid lesion development. On the other hand, in the lepramatous lesions Th2

cytokines were expressed and the lesions were considered to be as a result of multiplication of the pathogen at the local site due to active immunosuppression of bacillus reactive Th1 DTH CD4+ T cells by Th2 cytokines secreted by CD8+ T cells (Bloom et.al., 1992). Similarly, in the tuberculoid lesions of mycobacterial infection of humans (Dannerberg, 1991) as well as in experimental infection of mouse with M.*tuberculosis*, CD4+ Th1 responses are evident (Kaufmann, 1993). Such is the case with granulomatous lesion formation in listeriosis during reinfection. Accordingly, IFN–γ and TNF-α mRNA are abundantly present during reinfection (Ehlers et.al., 1992) and both of these cytokines are essential for granuloma formation (Buchmeir and Schreiber, 1985, Nakane et.al., 1988, Mielke et.al., 1992). Thus, the DTH response mediated by Th1 cells that is protective during primary infection and leads to remission of listeriosis, becomes pathological during reinfection resulting in tissue damaging granulomatous lesions.

The apparent question in T cell mediated IP is whether the effector T cells (CD4+ or CD8+) need to be physically present at the local site or can the pathology be mediated by their effector cytokines alone. The direct evidence for Th1 cytokines in inducing the tissue damaging inflammatory response could be found in transgenic animals engineered to express the cytokines in ectopic tissues (Taverne, 1994). Insulin dependent diabetes mellitus (IDDM) is considered as a T cell dependent auto immune disease mediated by Th1 cells. Although the disease is classified as an auto immune disorder due to pancreatic islet cell reactive T cell responses, viral etiology is suspected in the initiation of disease in humans (Gamble, 1980 and Notkins and Yoon , 1984). Nevertheless, when the Th1 cytokine IFN–γ was expressed as a transgene on the islet cells (Sarvetnick et.al., 1988), spontaneous development of IDDM was evident in the transgenic mice with associated inflammatory response. Other examples include the IFN–γ induced tuberculoid lesions (Nathan et.al., 1986 and Kaplan and Cohn, 1991) and cytokine neutralization experiments.

Although Th1 responses during primary as well as secondary infection are involved in immunopathological reactions in most bacterial and viral infections, Th2 responses also exert IP. In both RSV reinfection associated IP of lung (Graham et.al., 1993) as well as in schistosoma egg induced granulomatous lesions (Henderson et.al., 1992), Th2 cytokines, namely IL-4, IL-5 and IL-10, were spontaneouly expressed and IFN–γ mRNA expression was not seen. The direct role of Th2 cytokines in the

immunopathological reaction of RSV reinfection was evident in cytokine neutralization experiments in vivo. Accordingly, during RSV reinfection of FI-RSV immune mice, neutralization of either IL-2 or IFN–γ did not have any effect on the lung pathology. However, neutralization of either IL-4 or IL-10 markedly reduced the inflammatory response and the pathology was completely abrogated when both the cytokines were neutralized (Connors et.al., 1994).

Similarly, the granuloma formation in schistosoma egg induced pathology was considered to be mediated by Th2 cells. During initial phase of granuloma formation, both IL-2 and IL-4 are involved in the pathological process. Accordingly, *in vivo* neutralization of either IL-2 or IL-4 with specific anticytokine antibody therapy (Wynn et.al., 1992) abrogated granuloma formation. Conversely, injection of IL-2 enhances lesion size (Mathew et.al., 1990). On the other hand, unlike the granulomatous lesion development during reinfection with *Listeria* (Mielke et.al., 1992), or in tuberculiod lesions of mycobacterial infections (Nathan et.al., 1986), IFN–γ appear to play a very minimal role in the schistosoma granuloma formation and *in vivo* neutralization of IFN–γ did not exert any effect (Sher et.al., 1990). In contrast, IFN–γ is involved in the resolution of granuloma lesions. Macrophage activation results in IL-12 production and this cytokine was shown to be involved in differentiation of T cell precursors into Th1 effectors (Seder et.al., 1993 and Hsieh et.al., 1993). In this regard, administration of IL-12 in mice having established granulomas in schistosomal infection caused regression of granulomatous lesions with associated increase in Th1 cytokines and down regulation of Th2 cytokines, namely IL-4, IL-5, and IL-10 (Wynn et.al., 1994). These results directly implicate the Th2 cytokines in the granuloma formation and Th1 cytokine, IFN–γ, in resolution of lung pathology.

However, the pathological lesions mediated by Th2 cells were markedly different compared to Th1 cells. While the Th1 immunopathologial lesions were composed predominantly of macrophages and neutrophils characteristics of DTH response, the immunopathological leisons associated with Th2 immune response are dominated by eosionophils characteristics of allergic reaction. IL-4 dependent IgE production and mast cell degranulation is considered to be the cause of inflammatory response seen in parasitic IP and IL-5 is considered to be the key cytokine involved in maturation of circulatory and tissue eosinophils for induction of allergic reactions. Accordingly, anti-IL-5 treatment inhibits helminth induced eosinophilia (Coffman

et.al., 1989). However, abrogation of eosinophilia by anti-IL-5 treatment does not eliminate the egg induced granuloma formation in parasitic infections (Sher et.al., 1990). Thus, the granuloma formation appears to be a more complex process involving multiple components.

The Th1 and Th2 cytokines also exert a reciprocal negative regulatory function on each effector cell population. For instance, IFN–γ secreted by Th1 cells inhibit the growth of Th2 cells *in vitro*. Similarly, the Th2 cytokines, IL-4 and IL-10, inhibit the Th1 cells (Mossman and Coffman, 1989). The regulatory function of these cytokines are not only evident in *in vitro* experiments, but are also seen *in vivo*. In IFN–γ gene distrupted C57BL/6 mice, which are otherwise resistant to leishmaniasis, the susceptibility to infection was found to be associated with an increase in frequency of Th2 effectors rather than the absence of protective CD4+ Th1 response with static maintanence of Th2 effector population (Wang et.al., 1994). Similarly, in the reversal of schistosoma granuloma lesions following IL-12 treatment, the abrogation of pathology was associated with the induction of protective CD4+ Th1 responses rather than down regulation of pathological CD4+ Th2 cytokines alone (Wynn et.al., 1994). Similarly, in IL-10 (Kuhn et.al., 1993) and IL-4 gene distrupted mice (Kuhn et.al., 1991) have spontaneous manifestation of ulcerative enterocolitis due to over activation of Th1 cells. Nevertheless, the pathological mode of Th1 and Th2 cells vary at their effector level. In IP mediated by Th1 cells, concomitant with the tissue damaging inflammatory response, clearance or reduction in the load of infectious agent from the local site of infection is seen. Thus, in both primary as well as reinfection associated Th1 mediated immunopathological reactions, the cost of clearing the infectious agents by the normally protective CMI appear to be tissue damaging inflammatory response in certain locations. On the other hand, Th2 cell mediated IP persistence of antigenic stimuli due to the immunosuppression caused by Th2 cytokines (IL-4 and IL-10) impairs the clearance of infectious agents and results in persistence of antigenic stimuli (eg. Schistosomiasis).

However, it is interesting to note that unlike other viral and bacterial infections that tend to induce Th1 response, in RSV reinfection of the FI-RSV sensitized individuals, the pathogenesis appears to be mediated by Th2 responses (Connors et.al., 1994). Furthermore, the pathology is akin to that seen in parasitic diseases with profound eosinophilia of the lung with IL-5 induction along with other Th2 cytokines.

Although the reasons are unclear, as discussed earlier, at least the antigenic form of RSV used during initial priming appears to influence thecytokine induction pattern. In mice that are initially primed with vaccinia virus recombinants expressing RSV protein or live RSV, the pulmonary pathology during reinfection with live RSV was characteristic of Th1 mediated DTH response. In contrast, during reinfection of FI-RSV primed animal with live RSV, Th2 dependent IL-5 associated eosinophil infiltration in the lung was evident (Connor et.al., 1992 and Graham et.al., 1993).

The nature of Th1 or Th2 cell activation in response to infection is also influenced by the genetic make up of the host. The disease spectrum seen in inbred mice to certain diseases extends beyond the classical H2-linked immune response genes. The genetics of visceral lesishmaniasis caused by *L. donovani* has been well studied and the resistance and susceptibility to infection was found to be influenced by at least two genes, one of which is H2-linked (Blackwell et.al., 1985a). The second gene is the *ish* gene (Blackwell, 1985b). However, *ish* gene primarily regulates the ability of macrophages to control parasite multiplication, and mice that are homozygous for *ish* allele appear to produce primarily Th1 responses (Kaye et.al., 1991). Thus, both the genetic make up of the host as well as the causative pathogen appear to play a role in the IP.

The nomenclature of Th1 and Th2 is commonly used to describe the respective cytokines derived from T cells. However, it is now evident that these cytokines are also secreted by B cells, fibroblasts, monocytes, mast cells, as well as neutrophils. Since in inflammatory lesions invaribaly one or the other cell types are present and in addition to T cells, in analysing the cytokine responses in tissues or *in in vivo* samples a much broader term such as type I or type II is used in recent literature to represent the Th1 and Th2 cytokines, respectively. Even so, the identification of polarized cytokine secretion originally defined in CD4+ T cells has refined the modern concept in T cell immunobiology. Such refined approach of looking at T cells in terms of their effector cytokines helped to understand the various immunopathological conditions. With the perspective of host T cell derived cytokines role in various IP discussed above, in the following section the role of CD4+ T cells and their effector cytokines in mediating HSK are studied.

4. IMMUNOPATHOLOGICAL CONDITIONS IN HSV INFECTION

HSV infection is a common persistent viral infections in humans, and the pathological lesions are usually confined to mucocutaneous surfaces. Following primary infection, HSV undergoes an initial round of replication at the site of entry and reaches the nervous system *via* the sensory neurons in an anterograde fashion, and establishes latency in the neuronal tissues as well as in the local ganglion. Active viral replication is not evident at the latency site as evident from lack of viral gene transcription. However, periodic reactivation from latency results in recurrent episodes of clinical manifestations in the mucosal and cutaneous area. The mucocutaneous lesions are considered to be virological (Whitley, 1990) as evident from the recovery of infectious virus in lesions. However, a notion of immunopathological response cannot be ruled out. Exanthema formation seen in the base of the herpetic lesions is considered to represent in part the DTH reaction (Sell, 1987). However, the severity of the lesions is inconsequential and the lesions are usually controlled by antiviral drugs. On the other hand, a more serious complications of the herpetic infections occur in the ocular environment leading to permanent loss of vision. The lesions associated with herpetic infection of the eye is considered to represent IP, since they are readily amenable following immunosuppressive drug therapy, rather than by antiviral drug treatments.

Ocular HSV infections in humans are usually caused by HSV-1 (Hyndiuk and Glasser, 1986). In primary ocular HSV infections, the conjunctiva and the epithelium of the cornea are the common sites of infections and in immunocompetent individuals recovery from primary infections without any serious consequences to the ocular tissues (Daniels, 1982). Yet, a significant number of patients with primary infection experience recurrences (Kaufman, 1978) and reactivation of HSV from the latent infection was considered to be a causative factor in the ocular IP of HSV infections (Kaufman, 1978). Perhaps the most adverse effects of recurrent herpetic viral infections in humans are the ocular inflammatory response such as uveitis, retinal necrosis, and corneal stromal keratitis.

Uveal tract infections of humans by HSV is relatively uncommon. In animal models intravitreal inoculation of HSV antigens in rabbits recovering from primary infections with live HSV results in inflammatory response of the uveal tract (Oh, et.al., 1985). Although the immune elements involved in the secondary uveitis in response to

HSV antigens have not been studied, the secondary uveitis seen in response to HSV antigens are consistent with an immunopathological response. In this regard, efforts to recover infectious virus from the secondary uveitis lesions even during reinfection with live HSV were unsuccessful (Oh, 1976) and the effect of virus induced tissue damage was considered to be minimal. In addition, unlike primary uveitis induced with replicating viruses, secondary uveitis could also be induced with non infectious HSV antigens. Accordingly, in animals that are recovering from primary HSV infection, intravitreal inoculation of U.V. inactivated virus results in inflammatory response of the uveal tract infections. The inflammatory cells collected in the anterior chamber as well as in the uveal tissues are consistent with the clinical manifestations of the disease in humans. Auto immune response of CD4+ Th1 cells towards retinal S-antigen is also considered to be a factor in a rat experimental uveitis model (Gregerson, et.al., 1991). Although uveitis might represent a multiple etiological process in the pathogenesis, it is not known in HSV induced uveitis model whether primary ocular infection of live HSV also induces the exposure of retinal S-antigen to the immune system to mediate a multiple antigen associated immunolopathological consequence of uveitis.

Retinal necrosis is an another HSV induced ocular pathological condition in humans. In an experimental murine model, mild acute retinal necrosis associated with primary intraviteal inoculation of live HSV in the inoculated eye was considered in part as a result of initial viral replication. In euthymic mice, HSV inoculation in one eye results in retinitis in the uninjected contralateral eye between 8-10 days post inoculation with concomittant sparing of the retina of the injected ipselateral eye. The virus spreads through the CNS to the optic nerve and retina of the uninoculated eye, where acute retinitis progresses rapidly to retinal necrosis. Virus is cleared from all ocular and CNS sites of euthymic BALB/c mice by 21 day post inoculation. Although mild retinitis in the inoculated eye is due to initial viral replication at the local site, host CD4+ T cells are involved in the development of fulminant necrotizing retinitis in the uninoculated contralateral eye. In both euthymic and athymic BALB/c mice, comparable titers of virus are seen in the uninoculated contralateral eye at day 10 post inoculation. However, in euthymic mice, the virus is cleared from the local site in ensuing 4 days. Viral clearance is considered to be mediated by inflitrating CD4+ T cells, since persistence of virus is seen following depletion of CD4+ T cells. However, concurrent with clearance of virus from the contralateral uninoculated eye, retinal necrosis and

associated inflammatory response develop. The resulting necrotizing retinitis is considered to be immunopathological, since depletion of CD4+ T cells abrogates the response.

4. 1. Herpetic stromal keratitis

Herpetic stromal keratitis (HSK) is one of the ocular inflammatory responses manifested in the corneal stroma resulting in vision impairment and constitutes a major cause of non traumatic corneal blindness in humans. The initial damage to the corneal epithelium as a result of HSV infection is self limiting, and the lesions are resolved without any permanent damage. However, the frequent bouts of infections result in permanent damage to the corneal stroma leading to keratinization and scar tissue deposition with permanent vision impairment (Daniels, 1982 and Vaughan, 1989). The ensuing tissue damage at the site is considered to be primarily the result of host inflammatory response to the infection rather than due to cytopathic effects of the virus. Accordingly, antiviral therapy has no beneficial effect on the prognosis of HSK (O'Brien, 1986). On the other hand, HSK is readily amenable to corticosteroid therapy suggesting the immune factor involvement in the pathogenesis.

4. 1. 1. Murine models of herpetic stromal keratitis

In early animal models of HSK rabbits were used. However, much of the understanding of the pathogenesis of HSK stems from murine models. Although it is not absolutely required, the corneal epithelium is scarified prior to topical infection with infectious virus (Myers-Elliot et.al., 1983). During the early periods of viral infection, epithelial lesions are evident and the lesions are considered to be mediated by viral infection. Accordingly, antiviral drugs are effective in controlling the epithelial lesions and infectious virus is usually recoverable. However, in euthymic mice, stromal keratitis is observed in the ensuing several weeks. As will be discussed later, the stromal lesions are considered to be immunopathological responses mediated by CD4+ T cells. However, not all strains of inbred mice are susceptible to HSK. Some strains of mice, such as C57BL/6 and C3H are resistant, even though initial epithelial lesions were seen. Where as, in BALB/c and A/J mice, a characteristic HSK develops following topical infection with HSV(Opremcak et.al., 1990). Histologically, neovascularization of the corneal stroma, loss of epithelial integrity, and collection of

polymorphomononuclear (PMN) cells, lymphocytes, and macrophages in and around the capillaries are evident. During this period, lymphocytes are found in close contact with keratinocytes undergoing degenerative changes (Metcalf and Helmsen, 1977, and Metcalf and Kaufman, 1976). Later in the disease process, the degeneration of corneal layers increases and results in 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).

4. 1. 2. Evidence of CD4+ T cell mediated immunopathology in HSK

The most compelling evidence that inflammatory reactions in the cornea associated with HSV infection represents T cell mediated IP has come from studies in nude mice (Metcalf et.al., 1979, and Russell et.al., 1984). In athymic nude mice, corneal infection with HSV did not result in manifestation of HSK. Although during initial periods of infection, a superficial epithelial lesions were observed, it was subsequently cleared and the nude mice succumb to fatal viral encephalitis. On the other hand, the euthymic littermates developed characteristic HSK lesions (Metcalf et.al., 1979). Although the initial experiment suggested the role of T cells in the pathogenesis of HSK, direct evidence of T cells in inducing HSK was later shown by Russell and colleagues (1984). Accordingly, following reconstitution with HSV immune T cells prepared from euthymic BALB/c mice, HSV infected nude mice readily developed HSK.

The later studies were focused on identifying the T lymphocyte subset involved in the IP of HSK. Taking advantage of specific antisera capable of profoundly depleting one or the other two major subsets of T cells (CD4+ or CD8+), Newell and colleagues (1989a and 1989b) demostrated in BALB/c animal model that cells of the CD4+ T subset were essentially involved in HSK induced by HSV-1RE strain. In the absence of CD4+ T cells, HSK lesions failed to occur, whereas removal of CD8+ T cells actually resulted in more severe lesions. The role of CD4+ T cell subsets in the IP of HSK was later demonstrated in T cell deficient adult thymectomized BALB/c or SCID mouse reconstitution models (Doymaz et.al., 1992 and Mercadal, et.al. 1993). In these models, adoptive transfer of immune CD4+ T cells from the euthymic donor BALB/c mice readily resulted in HSK, while CD8+ T cells failed to do so. Additionally, by phenotypic evaluation of cells isolated from HSK lesions, presence of only CD4+ and

not CD8+ cells was noted in BALB/c animal models (Niemialtowski and Rouse, 1992). Although the implication of CD4+ T cell subsets in the IP of HSK was also shown by others (Hendricks and Tumpey, 1990), another school of thought also suggests that infection with HSV-1KOS strain may cause CD8+ T cell mediated HSK (Hendricks et.al., 1989a and 1989b). However, phenotypic evaluation of T cells isolated from HSK lesions induced by HSV-1KOS strain in BALB/c mice failed to confirm the role of CD8+ T cells in HSK (Niemialtowski and Rouse, 1992). Similarly, by adoptive transfer of HSV-1 KOS primed CD8+ T cells isolated from euthymic BALB/c mice into nude mice, Akova and colleagues (1993) failed to observe HSK manifestation. On the other hand, HSV-1 KOS immune CD4+ T cells readily manifested HSK in reconstituted nude mice. Although these results are in apparent conflict with the notion of virus strain influence in T lymphocyte subset selection in the IP of HSK (Hendricks et.al., 1989a and 1989b), perhaps the differences could be attributed to the mouse strains employed. Accordingly, in earlier studies in which CD8+ T cells were implicated in HSK by *in vivo* T cell depletion, A/J mouse model was employed. In contrast, in studies that failed to incriminate CD8+ T cells, BALB/c mouse model was used.

4. 1. 3. Proposed mechanisms of CD4+ T cells in the pathogenesis of HSK

Although CD4+ T cells have been implicated in mediating HSK lesions, several mechanisms by which the tissue damaging inflammatory response might occur in the cornea were put forth. Earlier studies suggested that the CD4+, MHC II restricted CTL observed in HSV infection in murine model might cause the direct tissue damaging inflammatory response by direct cytotoxic cell function. The presence of MHC II expressing cells in the limbus, and central and peripheral cornea in HSK lesions of human and mice (McBride et.al., 1988, and Opermcak et.al., 1990) favoured such a notion. Furthermore, keratinocytes treated with IFN–γ presented HSV antigens to CD4+ HSV specific CTL in chromium release assays. In this regard, the presence of CD4+ MHC II restricted CTL was demonstrated in the cervical and retropharyngeal lymph nodes of HSK mice and it was advocated that the direct cytotoxic function of CD4+ T cells might be a mechanism in the tissue damaging inflammatory response (Doymaz et.al., 1991). However, subsequent studies questioned such a notion. When the frequency of HSV specific CD4+ CTL isolated from the HSK ocular lesions were measured, quantitatively only as few as 20 CD4+ CTL precursors / eye were observed

(Niemialtowski and Rouse, 1992). Furthermore, the ocular cells were cultured *in vitro* prior to frequency analysis that impairs the quantitative estimation.

An alternate plausible mechanism of CD4+ T lymphocytes role in the IP of HSK was the DTH reaction. In the initial studies on HSK, Newell and colleagues (1989a and 1989b) observed significant HSV specific DTH response in mice experiencing HSK compared to parenterally immunized mice. Such severe DTH responses in HSK might indicate that the cells of CD4+ DTH phenotype are playing an important role in the manifestation of corneal pathology. In support to such a possibility, the histological appearances of the pathological cornea were reminescent of DTH reaction (Metcalf et.al., 1979 and Russell et.al., 1984). However, such a notion of DTH response in the cornea is not uniform. While one school of thought suggest that the DTH mechanisms mediated by CD4+ Th1 cells in HSK (Niemialtowski and Rouse, 1992 and Hendricks, et.al. 1992), an another school of thoughts arguethat the mechanisms could be similar to that of parasitic IP mediated by CD4+ Th2 cells (Jayaraman et.al., 1993).

The advocates of CD4+ Th1 cell involvement in the IP of HSK used two different approaches to arrive at the same conclusion. When the ocular cells were isolated from the HSK mice and stimulated *in vitro* with HSV or T cell mitogen, only the Th1 cytokine secretion was evident in the culture supernatant. The Th2 cytokines, such as IL-4 and IL-10, were not evident in the inflammatory cell isolates, although they were demonstrable in the splenic cells (Niemialtowski and Rouse, 1992). Similarly, by *in vivo* neutralization of the cytokines using anti-cytokine antibodies, it was found that neutralization of either IFN–γ or IL-2 during viral infection abrogated the HSK manifestation, while anti-IL-4 treatment was ineffective (Hendricks, et.al 1992). Although these direct experiments were indicative of CD4+ Th1 cells role in HSK, evidence to support the role of Th2 cells in HSK was also shown. In this regard, HSV glcoprotein D specific Th2 clones secreting IL-4 but not IL-2, TNF, or IFN–γ in response to antigenic stimulation induced HSK, when adoptively transferred into HSV infected recipients (Jayaraman et.al., 1993). Furthermore, the response of HSK development was rapid in reconstitution model (Jayaraman et.al., 1993). Although the evidence to demonstrate that the Th1 cells do not manifest the disease was not provided, the exacerbated manifestation of HSK with Th2 clones (Jayaraman et.al.,

1993) tend to argue against the findings of others (Niemialtowski and Rouse, 1992 and Hendricks et.al., 1992) on the role of CD4+ Th1 cells in the pathogenesis of HSK

5. RATIONALE FOR THE CURRENT STUDY ON THE HSK PATHOGENESIS

HSK represents a complex multistep disease process in which host CD4+ T cells appear to play an obligatory role in the pathogenesis. However, several issues regarding the pathogenesis of HSK remain ambiguous. As reviewed in the previous chapter, controversy still exists on the very basic aspect of whether the disease is potentiated by CD4+ Th1 or Th2 cells. Initial studies that proposed the role of CD4+ Th1 cells employed either anticytokine antibody therapy or *in vitro* stimulation of the ocular cells isolated during the clinical phase of HSK. Although in vivo neutralization of Th1 cytokines abrogated HSK, such neutralization was not effective when antibody administration was delayed (Hendricks et. al. 1992). In addition, the Th1 cytokine secretion from the ocular cells was detected after *in vitro* stimulation and may not represent the *in vivo* events (Niemialtowski and Rouse 1992). Furthermore, the conclusions were drawn from stimulating cells isolated during one particular phase of the disease progression and perhaps the early events during the initiation of HSK might be different.

In contrast, the contention that the CD4+ Th2 cells were involved in HSK pathogenesis were arrived at by adoptive transfer of HSV peptide specific CD4+ Th2 cells into the non immunocompromized recipients (Jayaraman et. al 1993). Thus, it is not known whether the manifestation of the disease is as a result of the transferred CD4+ Th2 cells or due to CD4+ Th1 cells of recipient origin. Normal progression of the disease in immunocompetent BALB/c mice is manifested around 15-20 days p.i. with HSV-1 at a time after clearance of virus had occurred (Metcalf and Kaufman, 1976). On the other hand, in the reconstituted mouse models, fulminant HSK occurs by 10 days following reconstitution with either polyclonal HSV immune (Mercadal et.al. 1993) or cloned CD4+ Th2 cells (Jayaraman et.al. 1993). The disease onset is earlier before virus clearance had been accomplished, and results in exacerbated lesions. In addition, adoptively transferred naive T cells also induced HSK in virus infected nude (Russell et.al., 1984) and SCID mice (Mercadal et.al., 1993). It is not known whether the naive T cells acquired HSV specific priming towards HSV derived antigens in the recipient environment or activated non specifically to mediate HSK. Thus, it became

questionable whether the manifestation of HSK in the reconstituted mouse model is mechanistically similar to the one seen in immunocompetent mice.

Furthermore, although initial assault with the viral infection is necessary to cause the disease, much of the damage is mediated by host T lymphocyte derived proinflammatory cytokines and the infiltrating inflammtory cells. While in active persistent viral infection it is concievable that the predominant T cell response may be directed towards the viral antigens, in HSK pathogenesis it remains arguable whether the CD4+ T cell response is in fact *solely* towards the viral antigen. As mentioned above, in immunocompetent mice, HSV is cleared from the local site by the time the disease is manifested and much remains in doubt whether the infiltrating CD4+ T cells during the clinical phase of HSK respond to persisting virus derived peptides or towards the cryptic antigenic determinants released from the immune sequestered cornea in response to initial HSV infection. The response of naive CD4+ T cells to induce HSK in virus infected nude (Russell et.al., 1984) and SCID mice (Mercadal et.al., 1993) tend to argue such an indirect mechanism.

Another unresolved issue is whether infectious, replication competent HSV infection is necessary to initiate the pathogenesis of HSK. Although T lymphocytes (CD4+ or CD8+) participate in HSK, infectious virus is undetectable at the inflammatory site, even though the disease is essentially initiated by the viral infection. Currently, it is thought that in immune privileged sites such as cornea, brain, or synovium pathogens such as viruses may provide the initial stimulus through increased presentation of sub-dominant determinants, either by molecular mimicry (Gammon et.al., 1991, Sercarz et.al., 1993, and Theophilopoulos, 1995) and/or by IFN-induced up regulation of gene expression for antigen presentation. Furthermore, once a response against a cryptic determinant has been initiated, there may be second waves of responses against additional cryptic determinants through the process of determinant spreading (Lehmann et.al., 1993). Since the CD4+ T cell mediated IP of HSK occurs in the immune privileged cornea, the question of CD4+ T cell activating determinants remain as a central issue in HSK pathogenesis.

Thus, the current study is guided by the hypothesis that both the cellular and molecular events behind the pathogenesis of HSK involves a complex multistep process initiated by HSV infection, which in turn causes an inflammatory response that

involves CD4+ T cells reactive with virus and perhaps virus induced proteins, which in turn participate in the inflammatory process or its resolution by the production of proinflammatory and regulatory cytokines.

6. SPECIFIC AIMS

1. To develop a technical approach that can quantitatively detect multiple cytokine mRNAs from small amount of *in vivo* samples, such as cornea, using Reverse transcription-Polymerase chain reaction (RT-PCR).

2. To measure *in vivo* expression of Th1 and Th2 cytokine mRNA during the course of HSK in immunocompetent BALB/c mice in both the immunopathological site (cornea) and the immune induction site (draining lymph node) to address the role of these cytokines in HSK pathogenesis without *in vitro* stimulation artifact.

3. To establish whether the IP of HSK in adoptive transfer models of SCID mice represents a disease which is mechanistically similar to that of HSK in immunocompetent mice.

4. To evaluate the role of CD4+ T cells reactive towards non HSV derived determinants in the pathogenesis of HSK.

5. To define the role of HSV *per se* in the initiation of HSK.

PART II

Development and Standardization of Quantitative Polymerase Chain Reaction for Quantification of Murine Cytokine mRNA Present in Small Number of Cells

1. ABSTRACT

Quantification of mRNA by polymerase chain reaction (PCR) is performed by using a competitor DNA standard in the PCR or by employing an internal standard RNA following simultaneous reverse transcription (RT) with the sample RNA. The latter approach is more reliable since it accounts for variations in both the RT and PCR steps. However, this section demonstrates that at times the internal standard RNA competes with the target mRNA in the PCR when both are present in disproportionate concentrations in the initial simultaneous RT reaction. To overcome the competition in the PCR, multiple simultaneous RT reactions with the sample RNA and the internal standard RNA are required. Such procedures make the approach time consuming and restrict the use of internal standard RNA for quantification of multiple mRNAs present in small amounts of sample RNA. These limitations are circumvented by the competitor DNA standard approach and mRNA levels can be quantified by calculating the RT efficiency. The situations are illustrated by quantifying the levels of IL-2, IL-4, IL-5, IFN–γ, and β-actin mRNAs in mitogen stimulated murine T cells using a multiple mRNA specific internal standard.

2. INTRODUCTION

The sensitivity of the polymerase chain reaction (PCR) allows the detection of low copy number mRNA transcripts following reverse transcription (RT) and the approach is finding myriad uses. Although valuable and easy to use as a qualitative assay, difficulties arise if precise quantification of target DNA or RNA is required. Variations encountered during the amplification process make inaccurate any comparison between a known amount of a standard template in one reaction with an unknown target template in another (Simmonds et. al., 1986). A subtle variation that affects the amplification rate 'R' that determines the yield 'Y' in the exponential phase of PCR will dramatically alter the reaction $Y = Yo\ (1 + R)^n$ where 'Y'o is the initial amount of template and 'n' is the number of cycles. To avoid such sample variations, both target and the standard templates should be coamplified together such that any variation in amplification will be proportional and quantification made more reliable.

Initial studies using quantitative PCR (qPCR) computed mRNA levels from a comparison with the coamplification of a known amount of β-actin mRNA or some other known template (Chelly et. al., 1988, Rappolee et. al., 1988 and Noonan et. al., 1990). Since such approaches are subject to competition between target and standard for the same reactants as well as amplification efficiency differences between disparate primer pairs, only imprecise results could be obtained.

To circumvent these problems, qPCR strategies have been devised originally by various workers (Becker-Andre and Hahlbrock, 1989, Wang et al., 1989 and Gilliland et al., 1990). Essentially, these methods are of two types. One that uses a PCR standard template that acts as a competitor with the cDNA to be quantified and the other employs a RNA standard. The RNA standard is simultaneously reverse transcribed with the sample RNA and co-amplified by PCR with the target mRNA specific primers. Thus the RNA standard serves as an internal standard both in the RT and PCR reactions. In both the approaches the target cDNA and the PCR standard are amplified with the same primers and the mRNA of interest is quantified in its cDNA form from a known amount of the standard.

However, the RNA standard approach was considered more precise since it accounts for the variations in both RT and PCR reactions. In this regard Wang and colleagues (1989) first reported a synthetic RNA standard containing a tandem array of 5' and 3' primers of multiple genes of interest. Subsequently, others (Kanangat et.al.,

1992, Funk and Fitzgerald 1991 and Bouaboula et al., 1992) have reported the use of similar synthetic RNA for qPCR. The design of the synthetic standard has been used in various gene expression studies (Wang et al., 1989, Funk and Fitzgerald, 1991, Bouaboula et al., 1992, Kanangat et al., 1992 and Platzer et al., 1992).

However, the experience with the internal standard RNA approach over 300 trials with the synthetic RNA standard described elsewhere (Kanangat et al., 1992) and in this section indicate the following. a) When an internal standard RNA method is used for quantification of mRNA, at times competition between the standard RNA and the target mRNA occurs in PCR making quantification labor intensive and expensive. b) This restriction demands a preliminary simultaneous RT-PCR titration study with the standard RNA and the sample RNA for each mRNA species in a sample to be quantified. c) Such preliminary steps warrant more sample RNA and restrict the approach where extensive quantification with a limiting sample source is desired. d) In contrast, the competitive PCR approach is a single step process allowing quantification of multiple mRNAs with a single RT reaction and a meaningful quantification of the mRNA can be performed by including the RT efficiency. With the approach, the output of four cytokines was quantified by resting and stimulated murine T cells and differences in regulation of IL-4 and IL-5 as compared to IL-2 and IFN–γ is demonstrated.

3. MATERIALS AND METHODS

3. 1. Cell culture and mRNA preparation

Splenic T cells from naive Balb/c and C57/BL mice were isolated by passage over nylon wool columns and cultured at a cell density of $1x10^6$/ml in RPMI[5% FCS(vol/vol)], L–glutamine (2mM), penicillin (100 U/ml), and streptomycin (100 μg/ml)]. Cells were stimulated with Con A (5 μg/ml) for variable periods after which cells were harvested and total cellular RNA was prepared using guanidium thiocyanate/CsCl as described previously (Chirgwin et. al., 1979). The total cellular RNA was then purified by phenol/chloroform extraction followed by ethanol precipitation and was quantitated spectrophotometrically.

3. 2. Construction of the internal standard for quantitative PCR

The internal standard DNA was synthesized by overlap extension PCR as described previously (Ho et. al., 1989). The design of the internal standard is shown in figure 1. The overall length of the internal standard was 561 bp and the primers were arranged in such a way that when any set of forward and reverse primers were used, the amplified internal control would always be of 281 bp product and not overlap in size with the coamplified cytokine gene of interest so as to be distinguished on an agarose gel. This internal standard template was referred to as UT-M.

3. 3. Reverse transcription and calculation of cDNA synthesis efficiency

one μg of total cellular RNA was reverse transcribed in a 20 μl standard reaction mixture using AMV reverse transcriptase (Promega). The cDNA synthesis efficiency was determined by trichloroacetic acid (TCA) precipitation assay using [α-^{32}P]dCTP (Ausubel et al., 1987). The efficiency of cDNA synthesis ranged from 23-27% and the mean of 25% was used in all the calculations.

3. 4. Quantitative polymerase chain reaction

Two methods were used. **1.** qPCR was performed by simultaneous RT of the target mRNA with an internal standard RNA. The internal standard RNA was synthesized from the linearized UT-M DNA template using T7 RNA polymerase by the standard *in vitro* transcription protocol (Promega). The resultant internal standard

RNA transcript was termed as SYNTA-M. The transcription mix was treated extensively with RQ1 RNAse free DNAse(Promega) to remove the template DNA and was measured spectrophotometrically. Thirty two to sixty four fmol of SYNTA-M was simultaneously reverse transcribed with 0.5-1 μg of total cellular RNA as described previously. Five μl of the resultant cDNA was serially diluted two fold and PCR was performed with 5 pmol of specific target cytokine primers. Twenty μl of each PCR reaction was electrophoresed in a 2% agarose gel and stained with ethidium bromide. The intensities of the bands were measured by video densitometry (Applied Imaging software system, Santa Clara, CA). To account for differences in the molecular weight of the target and the standard templates, the target band intensity was normalized to the internal standard intensity. The target cytokine mRNA present in the total cellular RNA population was quantified from the standard SYNTA-M curve by extrapolation as detailed previously (Wang et. al., 1989; Funk and Fitzgerald, 1991, and Kanangat et. al., 1992).

qPCR was performed by a competitive PCR approach using UT-M as a competitor DNA template as described previously (Gilliland et. al., 1990 and Platzer et al., 1992). Briefly, 0.5-1 μg of the total cellular RNA was reverse transcribed and the cDNA was diluted five fold. One to seven μl of the diluted cDNA was used for quantification of target cytokine mRNA with respective primers. A constant amount of diluted cDNA was placed in 8 or 9 tubes for PCR along with varying amounts of competitor (UT-M) template and quantification was done as illustrated in Fig.4. Briefly, the ratio of normalized target intensity to the competitor intensity was calculated and the log ratios were plotted against the concentration of competitor used in each tube in a log-log scale plot. The amount of target cytokine mRNA present in the volume of cDNA taken for quantitation was determined from the plot where the target/competitor ratio was 1. From the determined value the amount of target cytokine mRNA present in the total cellular RNA was calculated by correcting to 100% cDNA synthesis efficiency.

The PCR reaction was performed in 25 μl volume containing 1 x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% Triton X-100), 1.25 mM dNTPs, 1 U of Taq DNA polymerase, and 3-5 pmol of primers in a Perkin Elmer/Cetus thermal cycler for 25 to 30 cycles in the exponential phase. The PCR conditions involved denaturation at 94° C for 1 min 30 sec, annealing at 55°C for 2 min and

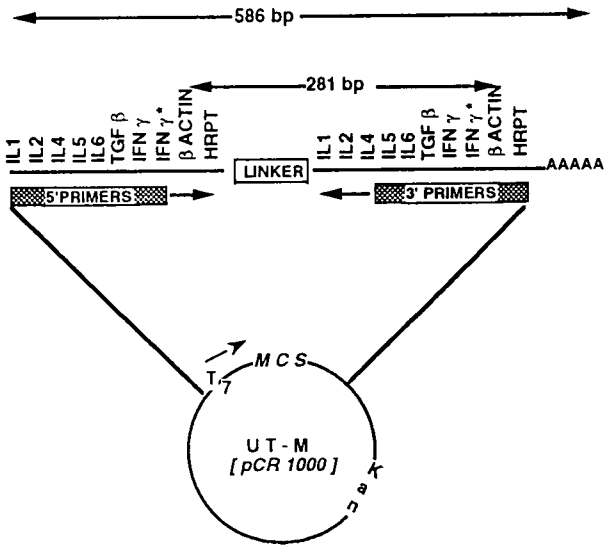


Fig.1

Design of UT-M

Primers were synthesized in oligonucleotide synthesizer and cloned into the multiple cloning site of PCR 1000 vector following PCR amplification by overlap extension. The T7 promoter on the vector was used in vitro for synthesis of the SYNTA-M RNA transcript. *Human cytokine sequences.

extension at 72^{o} C for 2 min. The reaction mixture was prepared as a master mixture to enhance the accuracy.

3. 5. Demonstration of primer efficiency in the amplification rate

Individual reactions were performed for different numbers of cycles with 10 pmol of β-actin, IL-2 and IL-5 primers and 10 pg of UT-M as a template. The amplified band intensities were determined as described earlier. The log intensity was plotted against the number of cycles in log Y = f(n) plot and the efficiency of amplification 'R' was calculated from the slope log(1+R). From the calculated 'R' value it is thus possible to determine the amount of amplified product at different number of cycles where the amplification is linear from the formula $Y=Yo(1+R)^{n}$ where 'Y' is the product, 'Yo' is the initial amount of template and 'n' is the number of cycles.

4. RESULTS

4. 1. Demonstration of the efficiency of primers in a PCR reaction

Of the many variables that make problematic the exploitation of PCR for quantitative analyses, differences in the efficiency of the primers used during amplification is the most important one. Primer efficiency can be defined as a function of a given primer set to give a maximum amplification of its template at the exponential phase of the PCR. The differences in amplification efficiency was demonstrated using b-actin, IL-2, and IL-5 primers as an example (Fig. 2). UT-M was used as a template to avoid any possible amplification differences in the template length between forward and reverse primers. The calculated amplification rate for β-actin was 47% whereas for IL-2 and IL-5 it was 39%. Although the percentage efficiency differences noted in this experiment were not constant and could be altered by varying the reaction conditions of the PCR, the magnitude of differential efficiency between different primer sets used in this experiment was similar.

4. 2. T cell cytokine quantitation using SYNTA-M RNA as a standard

SYNTA-M and total cellular RNA of different samples (Table 1) were simultaneously reverse transcribed. Five ml of the resultant cDNA was used in serial two fold dilution for the qPCR reaction as described in the methods. The normalized target intensity to the standard(SYNTA-M) was plotted against the concentration of SYNTA-M and the amount of target mRNA was calculated by extrapolation as illustrated for IL-4 mRNA quantification in 8 hr Con A stimulated T cells (Fig 3 A). The fact that the target and the SYNTA-M rate of amplification at each dilution was similar (approaches parallel) allowed quantification of the target mRNA in the initial RT reaction by extrapolation from the standard (SYNTA-M) straight line. The amount of target IL-4 mRNA was calculated as 6.6 fmol, and was in close agreement with the competitive PCR method of quantification described later (Table 1).

However, when the same amount of SYNTA-M RNA was used as a standard to quantify the target mRNAs present in various samples, a standard parallel straight line graph with a similar amplification rate at each dilution was difficult to obtain (Fig. 3B-D). The target IFN–γ mRNA in 12 hr sample was quantified using 64 fmol of SYNTA-M (Fig.3B). The plot implicated the preferential amplification of IFN–γ over the

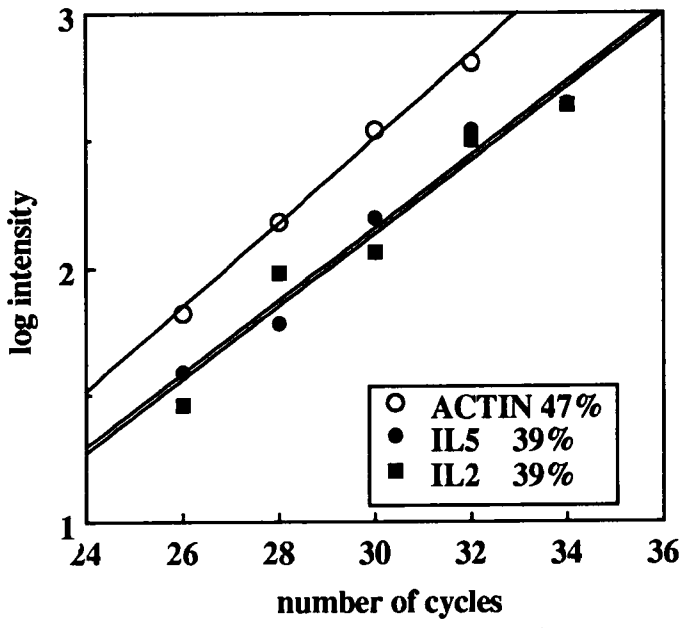


Fig.2.

Demonstration of primer efficiency in the amplification rate.

10 pg of UT-M template was individually amplified for different number of cycles with 10 pmole of β-actin, IL-5 or IL-2 primers and electrophoresed on 2% agarose gel. The amplified band intensity was measured as described in the methods and the efficiency of amplification 'R' was deduced from the slope log (1+R) of the plot log Y=log Yo + n log (1 + R). The linear regression of the lines were > 0.95.

Primers were as follows: β-actin, 5'GTGGGGCGCCCCAGGCACCA-3' (sense) and 5'CTCCTTAATGTCACGCACGAT-3' (antisense); IL-5, 5'ATGAGAAGGATGCTTCTGCAC-3' (sense) and 5'TCAGCCTTCCATTGCCAACTC-3' (antisense);IL-2, 5'ATGTACAGCATGCAGCTCGCA-3' (sense) and5' AGGGCTTGTTGAGATGATGCT-3' (antisense).

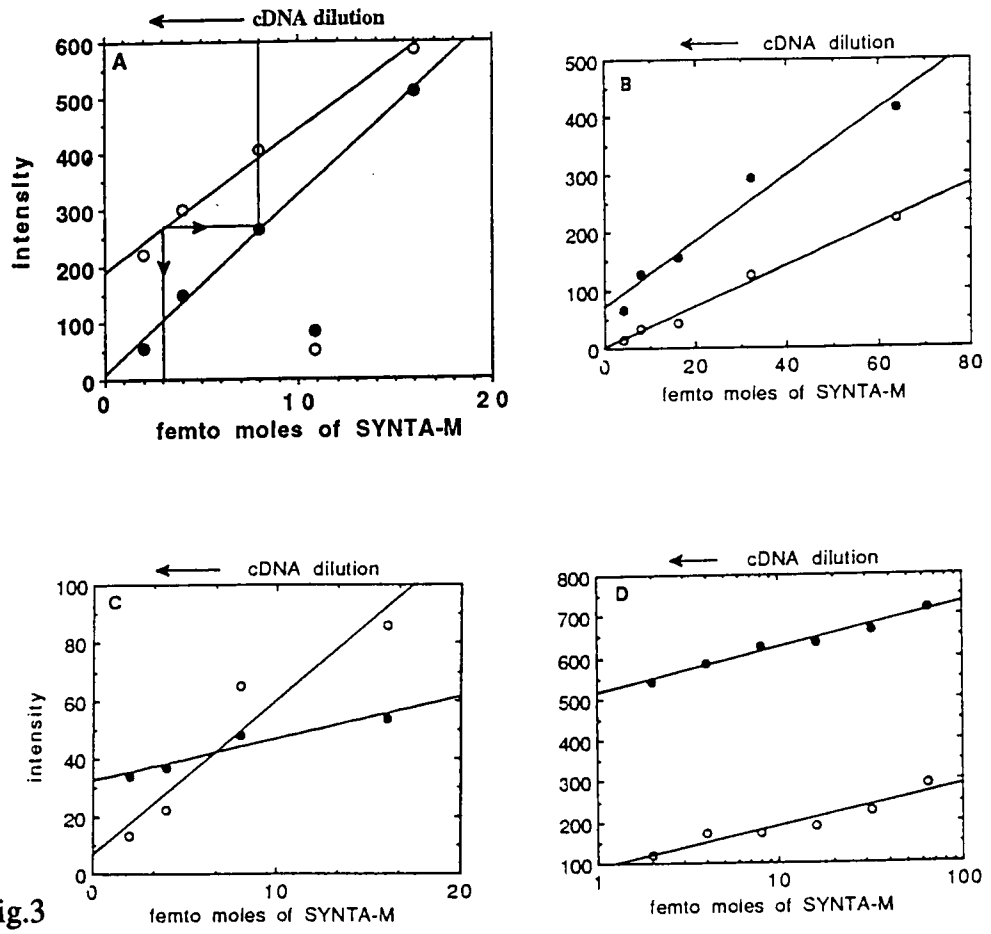


Fig.3 Quantitative plots using synthetic internal RNA standard.

0.5-1 μg of total cellular RNA of different samples were reverse transcribed with 32-64 fmol of SYNTA-M in a 20 μl reaction and 5 μl of the cDNA was used in 1:2 dilution. PCR was performed for 25 cycles with 5 pmol of respective primers. The normalized intensities were plotted in an arithmetic scale or semi-log plot against the SYNTA-M amount. (closed circle) target (open circle) SYNTA-M standard. (A) 8 hr Con A stimulated T cells total cellular RNA amplified with IL-4 primers. The amount of IL-4 at 1:2 dilution of the cDNA was calculated by extrapolation and the amount of IL-4 mRNA in 1 μg RNA was calculated with dilution factor as 6.6 f moles. (B) 12 hr (C) 1 hr and (D) 8 hr Con A stimulated T cell RNA measured for IFN–γ mRNA.

Primers: IL-4, 5'-ATGGGTCTCAACCCCCAGCTA-3' (sense) and 5'-CGAGTAATCCATTTGCATGAT-3' (antisense); IFN–γ, 5'-ATGAACGCTACACACTGCATC-3' (sense) and 5' – CAGCGACTCCTTTTCCGCTT-3' (antisense).

Table 1

Expression levels of IL-2, IL-4, IL-5, IFN–γ and β-actin mRNAs in (femto moles / μg) total cellular RNA of activated murine T cells

Time of stimulation	IL-2	IFN–γ	IL-4	IL-5	β-actin
Unstimulated	-	0.22	-	-	52.59 58.00*
1hr	0.03	0.32	0.32	0.002 0.008*	78.95
4hr	3.36	11.70	6.42	0.004	62.94 63.76+ 56.00*
8hr	62.83 56.37	47.87 54.69+	7.82 8.4+ 10.7*	0.01 0.016+	46.23 33.63*
12hr	26.48 27.48+ 28.19*	24.67 22.44*	0.78 0.79+	0.003	80.40
24hr	4.14	4.7 5.0+	0.14	0.001 0.008*	52.8 59.16* 60.92*

Balb/c T-cells were stimulated with 5 μg ConA/ml for indicated time. cDNA was prepared from 1 μg of total cellular RNA and the expression levels of cytokine and β-actin mRNAs were quantified by competitive PCR. Repetition on newly synthesized cDNA (*) and with the same cDNA (+) gave reproducible results with <4% variation. The data represents the sample 1 in Table 2.

SYNTA-M standard as the concentration of the latter diminished.

In contrast, when the same amount of SYNTA-M was used for quantification of IFN–γ mRNA in 1 hr stimulated T cells, the SYNTA-M standard was preferentially amplified at higher dilution over the target (Fig. 3C). Consequently, a precise quantitative plot could not be constructed. On the other hand, when the 8 hr stimulated T cell RNA was assayed for the IFN–γ using the same amount of SYNTA-M, the target IFN–γ and SYNTA-M standard were amplified at the same rate at each dilution (Fig.3D). Nevertheless, since the target amplification was greater than the standard at each dilution, extrapolation from the plot was not plausible. These effects (Fig. 3B-D) were seen consistently in repeated experiments even in higher primer concentrations and also in the analyses of TNF-α message in macrophages using similar other internal control RNA described elsewhere (Kanangat *et.* al., 1992) (data not shown).

4. 3. Quantitation of T cell cytokine mRNAs by competitive PCR using UT-M as a competitor

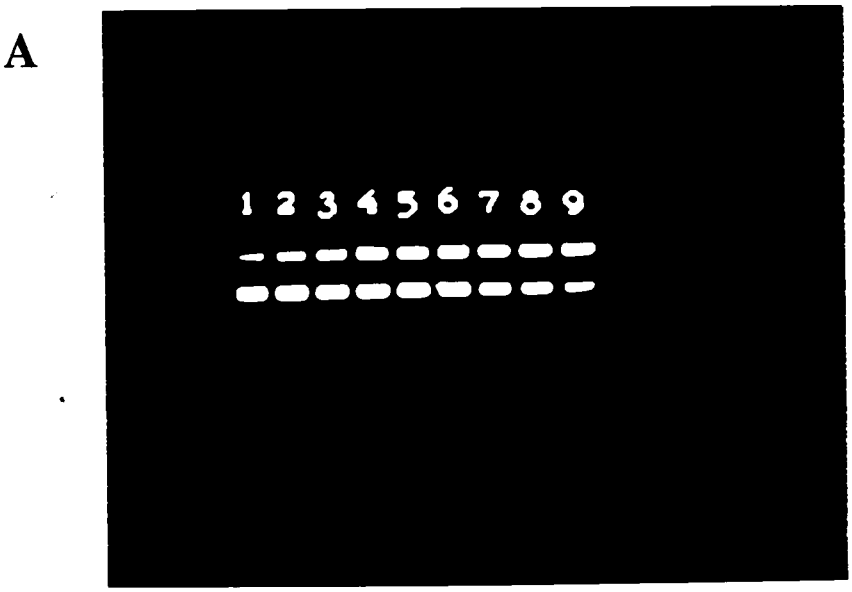
Due to the difficulty in obtaining a quantitative plot (as in Fig. 3 A) using SYNTA-M, T cell cytokine mRNA kinetic analyses were done by a competitive PCR method using UT-M as a competitor. The UT-M design allows it to be used as a competitor for various targets avoiding the creation of new competitor for each target. A constant amount of diluted cDNA was coamplified with varying amounts of competitor (UT-M) and the amplified products were distinguished in a 2% agarose gel. The normalized intensity ratios of the target and the competitor were plotted in a log-log or in an arithmetic scale plot. As expected, the plot was linear and the target mRNA present in the total cellular RNA was calculated where the target to competitor ratio was 1. A representative result of IFN–γ mRNA quantitation in 1 hr stimulated T cells is shown in fig. 4. The calculated value of the target was corrected for the dilution factor and multiplied by the cDNA synthesis efficiency factor for mRNA quantitation. The quantification results for IL-2, IL-4, IL-5, IFN–γ and β-actin mRNAs present in stimulated T cells at various time are summarized in Table 1. The proportions of these cytokines in relation to β-actin mRNA in Balb/c and C57/BL mice are summarized in Table 2.

Table 2

Proportion of IL-2, IL-4, IL-5, and IFN–γ mRNAs in relation to β-actin mRNA in Balb/c and C57/BL T cells

Cytokine		Naive BALB/c	Naive C57/BL		Naive BALB/c	Naive C57/BL
IL-2	1	--	--	2	--	--
IFN–γ	1	0.0004	0.002	2	0.001	ND
IL-4	1	--	--	2	--	--
IL-5	1	--	--	2	--	--
		4 hr stimulation				
		BALB/c	C57/BL		BALB/c	C57/BL
IL-2	1	0.005	0.27	2	0.16	ND
IFN–γ	1	0.18	0.14	2	0.12	ND
IL-4	1	0.1	0.01	2	0.04	ND
IL-5	1	0.0001	0.007	2	0.002	ND
		8hr stimulation				
		BALB/c	C57/BL		BALB/c	C57/BL
IL-2	1	1.52	0.6	2	0.67	ND
IFN–γ	1	1.035	0.6	2	0.874	ND
IL-4	1	0.22	0.2	2	0.1	ND
IL-5	1	0.0004	0.003	2	0.006	ND

Cytokine and β-actin mRNAs were quantified in 1 μg of total cellular RNA and compared with respective samples β-actin amount. ND - not done.



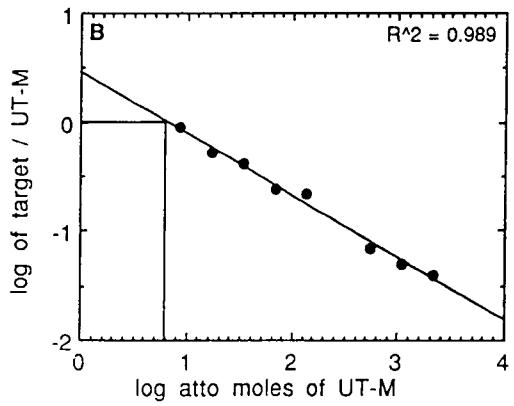


Fig.4

Representative gel (A) and graph (B) of the competitive PCR using UT-M as a competitor.

cDNA was prepared from 1 μg RNA of 1 hr stimulated T cells and amplified with IFN–γ primers. Five μl of 1: 5 dilution of the cDNA was coamplified with 1:2 serial dilutions of 273.4 atto mol of UT-M (lanes 1-9) as described in the methods. The log ratios of normalized intensities of the target and the UT-M (upper and lower bands respectively) were plotted on a log-log scale and the amount of IFN–γ was measured where the Target/UT-M ratio was 1. Three pmol of primers were used and PCR was performed for 30 cycles.

4. 4. Amplification rate of the target and the UT-M at equimole ratio

One important factor to be considered when using UT-M as a competitor is that both the length and the sequence differences between the competitor (UT-M) and the target do not alter the amplification rate at equimole ratios. In order to verify that the competitor and the target amplification rates were similar, calculated amounts of UT-M (281bp) to β-actin (581 bp) which provided a ratio of 1 in the quantification of unstimulated T cells were coamplified with the same amount of cDNA used earlier for different number of cycles with the β-actin primers. The amplification rates were determined from the plot logY=f(n) as described earlier. The amplification rates were exponential up to 28 cycles and attained a plateau after 30 cycles of amplification (Fig. 5). In the exponential phase of amplification (Fig. 5 inset) both the target and the UT-M amplification rates remained constant at an equimole ratio.

4. 5. Expression patterns of T cell cytokines

Following Con A stimulation of T cells for various time periods, all four cytokine mRNAs were maximally expressed at 8 hr and declined subsequently (Table 1). Strikingly, at 8hr the IL-4 and IL-5 mRNA levels were very low and constituted around 0.1% and 0.002% of the total cellular RNA and were in the proportion of 0.2 and 0.006 in relation to β-actin mRNA (Table 2) respectively. In contrast, IL-2 and IFN–γ mRNA levels constituted 1-2% of the total cellular RNA and were in the proportion of 0.6-1.5 and 0.6-1 respectively in relation to β-actin mRNA. Moreover, while the rise and decline of IL-4 mRNA was gradual with time, IL-2 and IFN–γ behaved identically with an initial sharp increase followed by a sharp decline. It is important to note that while no IL-2, IL-4, or IL-5 mRNA was detected in unstimulated cells, IFN–γ mRNA was seen in both the mouse strains.

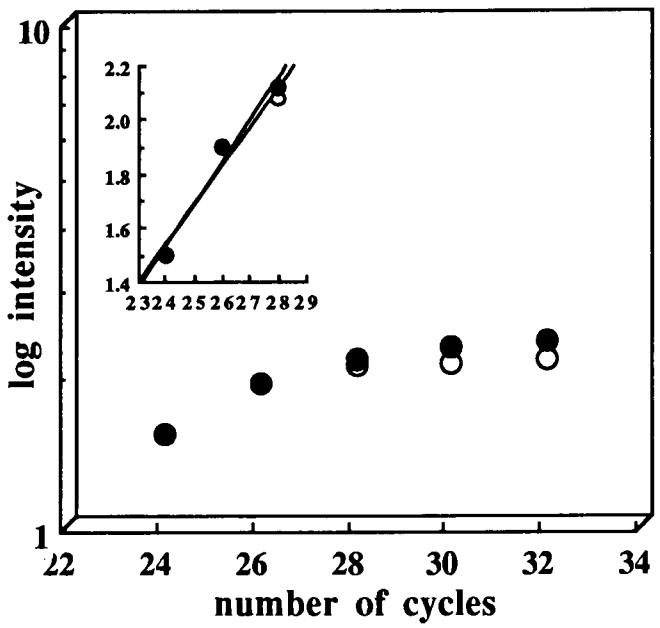


Fig.5

Demonstration of identical amplification rate of both target and UT-M at equimole ratio.

Different tubes containing 3 μl of 1:5 dilution of unstimulated T cell cDNA of 1 μg of total cellular RNA was amplified for various number of cycles with 623 atto mole of UT-M and 10 pmole of β-actin primers. The amount of cDNA and UT-M was derived from the calculation where the β-actin to UT-M ratio was 1 (not shown). The amplification was in exponential phase up to 28 cycles. (inset) exponential phase plot suggesting identical amplification rate. (closed circle) target (β-actin) (open circle) UT-M.

5. DISCUSSION

Although PCR is a powerful qualitative technique to detect DNA, or RNA following RT, quantifying the approach has proven to be a challenge. The most critical components in PCR are the primers and their efficiency of hybridization to their respective target sequences. Initial attempts of qPCR by coamplifying unrelated standards with target cDNA to avoid the variations in two separate reactions are judged unreliable because of differential DNA amplification contributed by the efficiency of disparate primer pairs in the reaction (Chelly et. al., 1988, Rappolee et. al., 1988 and Noonan et. al., 1990). Since the annealing kinetics of the complementary template strands and the primers to the template during PCR is influenced by the strand size and sequences (Wetmur, 1991), disparate primers yield differential amplification rates in proportion to their annealing efficiency (Saiki et. al., 1988). This issue as demonstrated here was addressed by others (Wang et al., 1989, Gilliland et al., 1990, Funk and Fitzgerald, 1991, Bouaboula et al., 1992 and Kanangat et al., 1992), who used the same set of primers to amplify target and standard to ameliorate the primer effect.

As regards, the concept of primer efficiency, several workers have derived qPCR methods where a known amount of standard and a target template to be quantified are coamplified using the same set of primers. One such method is that of competitive PCR described by Gilliland and colleagues (1990) and others (Bouaboula et al., 1992 and Platzer et al., 1992) for quantifying cDNA by PCR. The method exploited the principle that two templates upon coamplification compete for common substrates and preferentially amplify in PCR in proportion to their concentrations. In the competitive PCR approach, a simultaneous PCR occurred between a target and an added competitor standard template. Although the approach was shown valuable to quantify cDNA, the method fails to take account of RT variability.

An alternative strategy is the internal standard RNA method of qPCR where both the RT and PCR variations are taken into account (Wang et al., 1989, Funk and Fitzgerald, 1991 and Kanangat et al., 1992). In this approach, an internal standard RNA is added to the sample RNA and simultaneously reverse transcribed and quantified by PCR. The approach assumes the efficiency of RT of the standard and the target mRNA is similar in the simultaneous RT reaction and published reports (Wang et al., 1989, Funk and Fitzgerald, 1991 and Kanangat et al., 1992) attest to this point.

However, as shown in this section, with the internal standard RNA approach, at times quantification is difficult to perform. Plausible reasons for the difficulties in quantification could be a) lack of exponential phase b) differential amplification tendency of the target and the standard and c) competition between the target and the standard for amplification. Since the reactions were performed in the exponential phase, and the target and the standard had identical amplification rates at equimole ratios, the difficulties reported here, and mentioned by others (Funk and Fitzgerald, 1991 and Bouboula et al., 1992), were presumably due to competition between the target and the standard. Thus, if in the initial simultaneous RT, the chosen amount of RNA standard is in gross disproportion to the target mRNA of interest, both the target and the standard compete for amplification in PCR and preclude the construction of a quantitative plot. In this regard, the principle of competitive PCR becomes a limiting factor in the internal standard RNA approach. Some discordance, however, in the concentrations in the initial simultaneous RT can be tolerated. As demonstrated in our study of IL-4 mRNA quantification in 8 hr Con A stimulated T cells and in the published reports (Wang et. al., 1989, Funk and Fitzgerald, 1991 and Kanangat et. al., 1992) a window of disproportionate concentrations of the internal standard RNA and the target mRNA in the initial simultaneous RT did not exhibit competition in the PCR and allows the quantification of the target mRNA by extrapolation from the internal standard RNA.

The potential problem of competition inherent in the internal standard RNA PCR approach can be controlled by varying either the amount of sample RNA or the standard RNA in the initial simultaneous RT. However, it requires multiple RT reactions for each mRNA species to attain the optimum standard RNA to target mRNA ratio to avoid competition in the PCR. Consequently, it is expensive and labor intensive, as explained here and experienced by others (Funk and Fitzgerald, 1991 and Bouboula et al., 1992). Moreover, the approach is difficult when only minute amounts of sample RNA are available such as is the case with the biopsy specimens.

The advantage of the competitive PCR approach is that multiple mRNAs can be quantified in a single RT reaction. As demonstrated in the present study, the potential problem of RT variability could be minimized by actually measuring the efficiency of RT and using the value during the computation of the results. It is usually presumed that a qPCR standard should be close to identical in size and sequence to that of the

target to avoid differential amplification (Becker-Andre and Hahlbrock, 1989, Gilliland et al., 1990 and Li et al., 1991). However, the data demonstrate that such an effect does not exist when the templates are present at equimole amounts and that amplification is chiefly governed by the primers and the reactants. The study extends the observation of Bouboula and colleagues (1992) which demonstrated that size differences of 80 bases could be tolerated at equimole concentrations.

In conclusion, in this section a reliable means of quantifying multiple cytokine mRNAs using only limited biological samples is docimented. Since reproducible recovery of RNA even from the same number of cells is difficult to achieve, it was found convenient to normalize data in comparison to a housekeeping gene expressed in the same sample. With the approach, we could reproducibly measure levels of mRNA of 4 common $CD4^+$ T cell cytokine (IL-2, IL-4, IL-5, and IFN–γ) in normal and stimulated murineT cells.

PART III

Quantitative Measurement of *In Vivo* Expression of Th1 (Type I) and Th2 (Type II) Cytokine mRNA Expression During the Course of HSK in Euthymic BALB/c mice

1. ABSTRACT

Corneal infection with herpes simplex virus in mice induces an inflammatory response in the stroma (HSK) that appears to represent an immunopathological reaction in which T cells of the $CD4^+$ subset act as the essential participants. To assess the role of T cell cytokines at different clinical phases of HSK, corneas and draining lymph node (DLN) cells were collected and the levels of mRNAs considered as representative of type 1 and type 2 T cells were quantitated by RT-PCR. In the corneas collected prior to the onset of clinical disease, IFN–γ and IL-4 mRNA were detectable with IFN–γ 5-15 fold higher. During the onset and peak expression of clinical disease, type 1 cytokines IFN-γ and IL-2 were predominant in the corneas, and IL-4 levels were either very low or undetectable. Neither IL-10 nor IL-5 mRNA was present. After three weeks post infection when some animals with mild disease began to recover, high levels of type 2 cytokine mRNAs, particularly IL-10, were present. In addition, only during the recovery phase was IL-10 mRNA present in DLN samples. Levels of transcriptional activity for cytokine mRNAs during clinical HSK were higher in corneas than in the corresponding DLN samples. The results indicate that IL-10 may be involved in HSK resolution and that the stimuli for cytokine induction in the cornea may differ from those in the DLN.

2. INTRODUCTION

One of the more significant effects that infection with herpes simplex virus (HSV) induces is an inflammatory response within the corneal stroma of the eye. This all too common cause of vision impairment is considered to represent an immunopathological response to virus (Doymaz and Rouse, 1992). From the studies in mouse models designed to elucidate the pathogenesis of herpetic stromal keratitis (HSK), it is evident that the disease only occurs in euthymic animals (Metcalf et.al., 1979 and Russell, et.al., 1984). Athymic and SCID mice, which normally fail to express HSK, readily do so if given adoptive transfers of T cells (Russell et.al., 1984 and Mercadal et.al., 1993). Lesions usually persist well beyond the time when infectious virus can not be recovered from the inflamed tissues. Although there is some disagreement about the identity of T cells that are principally involved in the immunopathology, most investigations have implicated a pivotal role for $CD4^+$ T cells in mediating HSK (Newell et.al., 1989a and 1989b, Hendricks and Tumpey, 1990, Niemialtowski and Rouse, 1992, Hendricks et.al., 1992).

At least two groups have advocated that T cells with a type 1 cytokine profile (Th1) mediate the pathology (Niemialtowski and Rouse 1992 and Hendricks et.al., 1992). In contrast, on the basis of adoptive transfer experiments with a HSV specific clone, another group of investigators suggest that cells of the type 2 cytokine profile contribute to lesions (Jayaraman et.al., 1993). However, HSK as with other chronic inflammatory responses, is a dynamic process and the balance between various cell types likely changes with time. In this section, the issue of cytokine dominance is readdressed in HSK during the course of the disease and the levels of cytokine mRNAs were quantified by a sensitive quantitative RT-PCR approach (Gilliland et.al., 1990, Kanangat et.al., 1992, and part II). Accordingly, the results measure which cytokine genes are transcriptionally active at the time of sacrifice. The results demonstrate that the profile of cytokine expression changes during the course of infection. Initially type 1 cytokines, particularly IFN$-\gamma$ are predominant, but as the disease regresses type 2 cytokines become prominent and could be associated with lesion remission.The results are discussed in terms of approaches likely to prove useful to modulate the expression of HSK and to further understand the mechanism of its pathogenesis.

3. MATERIALS AND METHODS

3. 1. Mice

Four to five weeks old male or female BALB/c mice (Harlan Sprague Dawley, Inc. Indianapolis, IN) were used for the experiments. The animals were acclimated for a week before use in specific pathogen free animal colony accredited by the American Association for Accreditation of Laboratory Animal Care. All experimental procedures were conducted according to the association for research in vision and ophthalmology (ARVO) resolution on the use and care of laboratory animals.

3. 2. Corneal infection and clinical observation

Corneal surface of the deeply anesthetized mice (methoxyflurane [Metafane]; Pittman-Moore, Mondelein, Ill.) was scarified with a sterile 27–gauge needle and 5 x 10^6 $TCID_{50}$ HSV-1/ RE strain grown on vero cells was applied in 4 µl volume and gently massaged with the eye lids. Control animals received PBS on the scarified cornea. Animals were examined at different days post infection (p.i) by slit lamp SL5 biomicroscope (Kowa Co., Nagoya, Japan) and the severity of the stromal keratitis was recorded with the following scoring system as described elsewhere (Newell et.al., 1989a and 1989b): (0), clear cornea; (1+), mild corneal haze; (2+), moderate corneal opacity or scaring; (3+), severe corneal opacity with visible iris; (4+), opaque cornea, iris not visible; (5+), necrotizing stromal keratitis.

3. 3. Isolation of corneal specimens for RNA preparation

After different days p.i., the corneal pathology was scored as described earlier (Newell et.al. 1989 a and 1989b)) and the corneas and the cervical and retropharyngeal lymph nodes were isolated. The corneas were excised free of the scleral tissues and cut into fine pieces and used directly for RNA isolation. Ten mice per group were used for the preclinical phase and sacrificed at 5 and 7 d p.i. For the onset phase samples, 20 mice were infected and the animals that exhibited 1+ clinical score were used. For the clinical and resolution phase samples 30-40 mice were used. The clinical phase samples were collected from the animals manifesting 3+ and 4+ clinical score. Some animals developed necrotizing lesions (5+) and were excluded from analysis since RNA was either non-recoverable or of poor quality. The resolution phase samples were

collected after three weeks of infection at a time when around 20% of animals in other experiments underwent complete clinical resolution.

3. 4. RNA preparation

All reagents and materials used for handling of the RNA samples were rendered RNAse free by pretreatment with 0.1% diethylpyrocarbonate as per the standard protocol (16). Total cellular RNA was prepared from the minced corneas and the DLN cells by RNAzol-B (Cinna/Biotec Laboratories International). In the preclinical and onset phase DLN samples where cell yield was high due to large sample size, RNA was isolated by guanidium thiocyanate/Cesium chloride centrifugation method described elsewhere (Chirgwin et.al., 1979) followed by phenol-chloroform extraction and ethanol precipitation. The samples were stored as lysate at -80°C and the RNA was isolated a week prior to the analysis. The RNA samples were stored at -80°C until use (< 3 weeks) or used immediately for cDNA synthesis.

3. 5. Reverse transcription

The total cellular RNA was heated at 68°C for 2 min. and reverse transcribed in a 20 µl reaction mixture containing 1X reaction buffer (10mM Tris-HCL, pH 8.8, 50 mM KCL, and 0.1% triton X-100), 5mM MgCl2, 2mM dNTP mixture, 40 U RNAsin, 1 µg of oligo dT primer, and 15 U of AMV reverse transcriptase (Promega), at 42°C for 1 hr. The cDNAs prepared from the corneal and DLN samples were diluted 1:3 in DNAse free water to obtain a sufficient handling volume and heated at 99°C for 5 min. and used in the PCR reaction.

3. 6. Polymerase chain reaction (PCR)

PCR was performed in a 25 µl reaction mixture containing 1 X PCR buffer (10mM Tris-HCL, pH 8.3, 50 mM KCL, 2 mM $MgCl_2$, 0.01% Triton X-100), 200 µM dNTP mixture, 1 U of Taq DNA polymerase (GibcoBRL), and 2-3 pmol of primers in a PTC-100 (M J research, Inc., MA) thermal cycler. PCR conditions involved denaturation at 94°C/ 1 min. 30. sec., annealing at 55°C/2 min., and extension at 72°C/2 min. The reaction mixture was prepared as a master mixture to minimize the reaction variation.

3. 7. Qualitative PCR analysis of the cytokine mRNA expression

A portion of the cDNA preparation of each experimental sample was used initially in the qualitative PCR to analyze the expression pattern of the cytokine mRNA of interest. Individual PCR was carried out using equal volume of cDNA for 35 cycles with specific primers for each cytokine mRNA regardless of the exponential phase to analyze the mRNA expression in an all or none fashion as well as to assess the amount of competitor to be used for quantitative PCR. The detection limit of mRNA molecules at 35 cycles with the primers listed in table 1 is ~< 100 molecules as determined in preliminary experiments. The expression levels of the mRNAs present in each cDNA sample were then quantified using an internal standard in the quantitative PCR described below. RNA from the conA stimulated T cells was used as a positive control.

3. 8. Primers for the PCR

Cytokine mRNA specific primers were designed from published cDNA sequences. The primers were positioned to span the full length mRNA sequences so that the fully spliced mature mRNA product could be distinguished from the unspliced mRNA product in an agarose gel. The primer sequences and their amplification product size of the fully spliced mRNA are listed in table 1. The specificity and the efficiency of the primers were tested in the preliminary experiments (part II)

3. 9. Internal standard for quantitative PCR

Synthetic internal standard containing tandem array of 5' and 3' primers of different cytokines was synthesized by overlap extension PCR (13) and cloned into the pCR 1000 TA vector (Invitrogen). The primers were positioned in such a way that the internal standard would be of different size (281 bp) when coamplified with the specific target cytokine mRNA of interest so as to be distinguished on an agarose gel from the target cytokine mRNA. The design of the internal standard and its application to measure cytokine mRNAs were described in detail previously (part II).

3. 10. Quantitative PCR

To circumvent amplification differences contributed by the primers (Gilliland et.al., 1990, Kanangat et.al., 1992, and part II) and to compare the relative levels of

Table 1

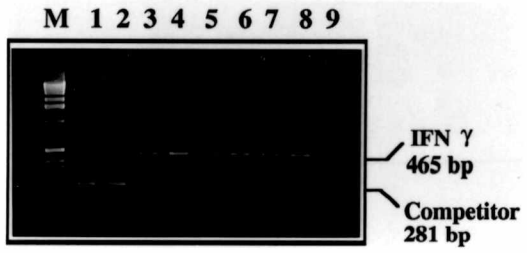
The sense and antisense primers of cytokine mRNAs

mRNA species	Sense (5'---3')	Amplified Product Size (bp)
IL-2	ATG AGA AGG ATG CTT CTG CAC	510
IL-4	ATG GGT CTC AAC CCC CAG CTA	420
IL-5	ATG AGA AGG ATG CTT CTG CAC	402
IL-10	ATG AAATAT ACA AGT TAT ATC	537
IFN–γ	ATG AAC GCT ACA CAC TGC ATC	465
β-actin	GTG GGG CGC CCC AGG CAC CA	548
	Antisense (5'---3')	
IL-2	AGG GCT TGT TGA GAT GAT GCT	
IL-4	CGA GTA ATC CAT TTG CAT GAT	
IL-5	TCA GCC TTC CAT TGC CAA CTC	
IL-10	TTA GCT TTT CAT TT GAT CAT	
IFN–γ	GCA GCG ACT CCT TTT CCG CTT	
β-actin	CTC CTT AAT GTC ACG CAG GAT	

The sense and antisense primers of the mRNAs were derived from the published sequences and were used to construct the competitor template (part II) by positioning them in a tandem fashion as mentioned in the materials and methods. The primers amplify the competitor (281 bp) and the given mRNA species in different sizes upon coamplification and the products are distinguishable in 2% agarose gel.

expression of different mRNA species within the sample, the synthetic competitor described above was used as a standard in the competitive quantitative PCR reaction. Briefly, a constant amount of the sample cDNA was coamplified with the specific cytokine primers in 6-8 reactions along with varying amounts of the competitor Following amplification at the exponential phase, the competitor and the target cytokine products were separated in a 2% agarose gel and stained with ethidium bromide. The intensities of the amplified products were measured by the video densitometry (Applied Imaging Software System, Santa Clara, CA). To account for the molecular weight difference in intensities, the target intensity was normalized to the competitor intensity and the log ratios of the normalized target intensity to the competitor intensity were plotted in a log-log scale plot. The amount of the target cytokine mRNA of interest present in the volume of cDNA taken for quantification was determined from the plot where the target/competitor ratio was 1. All the cytokine mRNAs were quantified from the same cDNA pool to avoid any sample variation. The target mRNA molecules (copies) were calculated by Avogadro values (6.023×10^{23} molecules/mole). A representative quantitative PCR is described in Fig. 1(A-B). The cytokine mRNAs were quantified along with the β-actin mRNA in each sample. Expression of β-actin mRNA served as a control both for the RNA preparation as well as for temporal comparison of the different mRNA species across the samples. PCR was carried out for 30-35 cycles.

A



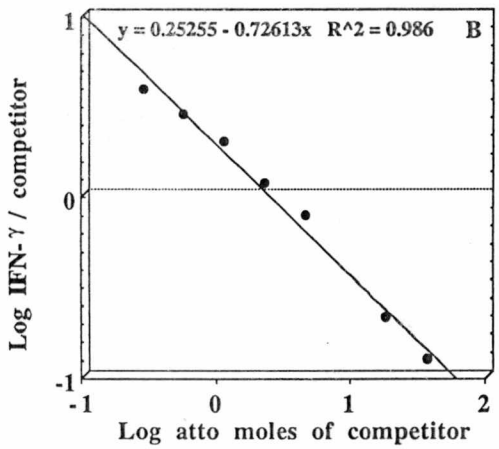


Fig. 1

Quantification of cytokine mRNAs by competitive quantitative PCR.

(A) Representative quantitative reaction of IFN–γ using the competitive standard. A constant amount of cDNA was coamplified with varying concentrations of the competitor standard (lanes 1-8) and (B) the log ratios of the amplified target cDNA and the competitor intensities were plotted against the concentration of the competitor used in each reaction. The amount of mRNA present in the initial volume of cDNA was calculated from the plot where the amplification ratio was 1 (representing lack of competition and identical amplification rate (part II). The deviation from the linear regression of 1 represents the error values. All regression values of the plot for the mRNA levels summarized in table 2, 3 and 4 were >0.93 (maximum error value of 7%). lane 9; negative control M; 1Kb marker.

4. RESULTS

4. 1. Course of HSK in immunocompetent BALB/c mice

Following infection of the scarified cornea with the RE strain of HSV-1, the majority of eyes developed lesions but the severity and time of onset varied. In some animals, the onset was evident at day 10, whereas in others, lesions were not apparent until day 16-19. After onset, lesions usually progressed in severity over the ensuing 5-7 days (clinical phase). Subsequently, events varied, with some animals developing necrotizing lesions, and the others remaining clinically unchanged. Severity of lesions did not correlate with the time of onset of visible lesions. In some animals (15-25%) lesions declined in clinical severity with resolution completed within 10 days (resolution phase). In animals that resolved, the severity of lesions at their peak was moderate. In one experiment involving 26 animals, 22 developed clinical lesions and 6 of these had resolved their lesions by 4 weeks. Eight of the clinically affected animals developed severe necrotizing lesions. Corneas and DLN (pooled samples) cells were collected at the various phases of HSK for the quantification of type 1 (IL-2 and IFN–γ) and type 2 (IL-4, IL-5 and IL-10) cytokine mRNAs.

4. 2. Cytokine mRNA expression in corneas during different clinical phases of HSK

As is evident in table 2, the pattern of cytokine mRNA expression varied with the course of HSK. In samples taken from naive animals, neither type 1 nor type 2 cytokine mRNAs were detectable. However, minimal levels of IFN–γ, but not other cytokines, were detectable in 5 days post-mock infected (trauma control) samples but not at later time periods. Maximal levels in trauma samples were less than 1000 copies/cornea and never exceeded 0.03% of β-actin mRNA levels detected in the same samples. In corneal samples taken from virus infected mice prior to the onset of clinical lesions, two cytokine mRNAs (IFN–γ and IL-4) were detectable. However, the expression of IL-4 was variable. When IL-4 and IFN–γ mRNAs were coexpressed, the copy numbers of IFN–γ were 5-15 fold higher than IL-4. Only the preclinical samples in which both the cytokine mRNAs were found are presented in table 2.

A dominance of type 1 cytokines was clearly evident in corneal samples collected at onset and during the clinical phase. Accordingly, IFN–γ and IL-2 mRNA

Table 2

Expression levels of cytokine mRNAs in the cornea
during the course of HSK
mRNA copies (x1000) per cornea

mRNA Species	Trauma Control	Preclinical 5d	Preclinical 7d	Onset	Clinical Phase	Resolution Phase
Score		0	0	1+	3+/4+	2+/3+
IFN–γ	1[a]	35	120	796	951	674
IL-2	NE[b]	NE	NE	83	64	Trace
IL-4	NE	7	7	Trace[c]	Trace	280
IL-10	NE	NE	NE	NE	Trace	158
IL-5	NE	NE	NE	NE	NE	NE

10 corneas were isolated during the course of HSK and total cellular RNA was isolated as described in the materials and methods. The cytokine mRNA expression was analyzed qualitatively and was quantified from the cDNA prepared from the total cellular RNA isolated from the pooled samples as in Fig. 1 (A & B) and the cytokine levels were expressed per corneal basis.

[a]weak expression of IFN–γ mRNA was routinely noticed and was quantifiable only from large samples of pooled RNA.

[b] not expressed;

[c]weak amplification was evident in the qualitative RT-PCR, but fell below quantifiable levels. The preclinical, onset, and clinical phase samples were analyzed by RT-PCR in five independent experiments and quantified in three samples. The resolution phase samples were analyzed in three experiments and quantified in two. Decimals were rounded to full figures.

were readily detectable, but IL-4 was either undetectable or expressed at too low levels to accurately quantify. Neither IL-5 nor IL-10 mRNA was present. The fact that levels of both IFN–γ and IL-2 mRNA were at their peaks during the onset and clinical phases supports the work reported by others, observed from the *in vitro* stimulated cultures, that HSK represents a disease primarily mediated by CD4+ T cells of type 1 phenotype (Niemialtowski and Rouse, 1992). Examination of cytokine mRNA levels in the corneal samples collected from animals after 3 weeks p.i., when some animals ultimately resolved their lesions showed a significantly different cytokine mRNA profile. In animals examined in this category, the clinical severity at their peak was moderate (1+-3+). In such samples, type 2 cytokine mRNAs were abundantly present and type 1 cytokines were actually declining. IL-2 became almost undetectable and was not present at sufficient levels for quantification. IFN–γ mRNA was still present in high amounts, although less than that of the clinical phase. The levels of IL-4 mRNA were 30-40 fold higher in the resolution phase than that was found in the preclinical samples. Of particular interest was the detection of high levels of IL-10 mRNA in the resolution phase samples. Despite the presence of the type 2 cytokines, IL-4 and IL-10, IL-5 mRNA remained undetectable in the corneal samples.

4. 3. Expression of cytokine mRNAs in the DLN:

In DLN samples (table 3 and 4) collected from animals at the same time as corneas, it was possible to detect both type 1 and type 2 cytokine mRNAs. However, only at the resolution phase were quantifiable levels of IL-10 mRNA present. In the trauma control samples, as in the corneas, only IFN–γ mRNA was found. It is evident that at all the phases, type 1 cytokines predominated and the pattern as well as the levels of expression did not fully correspond with those found in the corneas. For example, in the preclinical phase corneal samples (table 2), minimal IL-4 but not IL-2 mRNA was found, but in the DLN samples IL-2 mRNA levels were 10-20 fold higher than IL-4. Also, at the clinical phase, the cornea showed only a type 1 cytokine profile, but in the DLN, type 2 cytokines were spontaneously expressed. The data may serve to indicate that the population of cells in the inflamed corneas differ functionally from those in the DLN.

Table 3

Expression levels of cytokine mRNAs in the draining lymph nodes

during the course of HSK

mRNA copies ($x10^5$)/μg of total cellular RNA

mRNA Species	Trauma Control[a]	Preclinical (7d)	Onset
IFN–γ	13	262	2586
IL-2	Trace[b]	653	1900
IL-4	Trace[b]	31	52
IL-10	NE[c]	Trace	Trace
IL-5	NE	15	18

Total cellular RNA was isolated from the DLN cells and the expression levels of cytokine mRNAs were quantified in the cDNA samples prepared from 1 μg (1-2 million cells) of RNA.

[a], in both naive (data not shown) and trauma control DLN cells frequently only IFN–γ mRNA expression was evident. Only the 5d trauma control DLN sample was documented so as to be in correlation with the trauma corneal sample (table 1).

[b], weak levels of amplification below quantifiable levels.

[c], not expressed.

Table 4

Expression levels of cytokine mRNAs in the draining lymph nodes during the course of HSK

mRNA copies ($x10^3$)/one million cells

mRNA Species	Clinical Phase	Resolution Phase
IFN-γ	144	1149
IL-2	44	98
IL-4	16	43
IL-10	Trace[a]	69
IL-5	14	NE[b]

Cytokine mRNA levels expressed in one million DLN cells were quantified as mentioned earlier. [a], weak non quantifiable level of amplification, [b], not expressed.

4. 4. Change in the expression levels of cytokine mRNA in the cornea and the DLN during the course of HSK

To gain insight into the in vivo transcriptional levels of cytokine mRNA, the quantifiable amounts of cytokine mRNAs present in the cornea and the DLN during different phases of the disease were expressed as a percentage in relation to β-actin mRNA (Fig 2). Such an approach allowed the comparison of the expression levels between the cornea and the DLN regardless of the absolute number of molecules present or the number of corneal or DLN cells used for RNA isolation. The results show some interesting differences between the corneal and the DLN samples during the course of the disease. During the early phase, that plausibly represents the priming response, the percentage expression of cytokine mRNA in DLN (Fig. 2A inset) samples was higher than that in corneas (Fig. 2A). However, at later phases, a reverse pattern was evident. Accordingly, during the onset (Fig. 2B), clinical (Fig. 2C) and even in the resolution phases (Fig. 2D), the percentage expression of detectable cytokines in the corneal samples was far higher than that in the DLN (Fig 2B-D insets). The higher transcriptional activation seen in the corneal cells may either represent a different functional population than that is found in the DLN or that the activating stimulus is different for the corneal and the DLN cells.

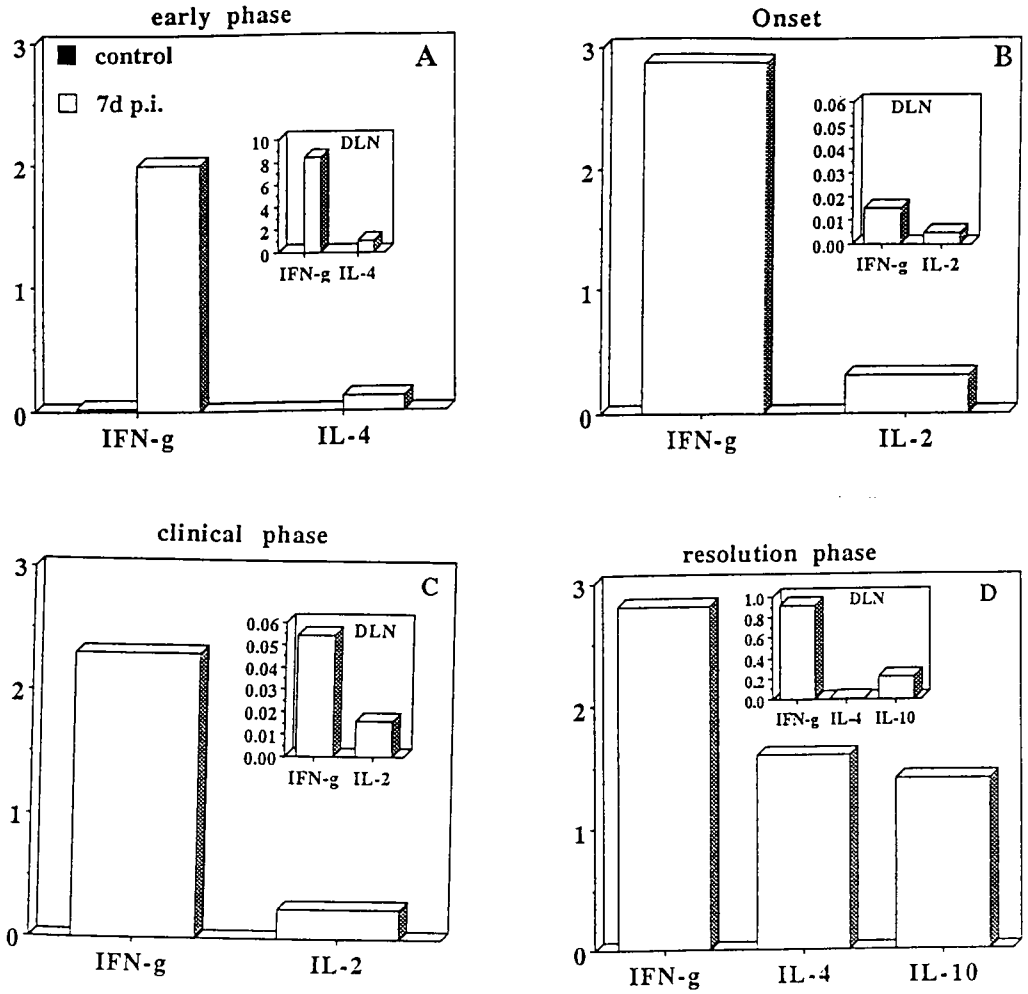


Fig. 2.

Change in the temporal frequency of cytokine mRNAs transcription during the course of HSK.

The levels of cytokine mRNAs expressed in the corneal samples were compared with the corresponding DLN cells in relation to the quantified levels of β-actin mRNA

$$\left[\frac{\text{cytokine mRNA copies}}{\beta\text{-actin mRNA copies}}\right] \times 100$$

in early (A), onset (B), clinical (C), and resolution phase (D) corneal samples and the DLN samples (insets)

5. DISCUSSION

In this section, by using a sensitive competitive RT-PCR approach the levels of type 1 and type 2 cytokine mRNAs at various stages during the development of an inflammatory disease of the cornea (HSK) caused by infection with HSV was quantified. The results demonstrate that the profile of cytokine expression changes during the course of infection. Initially type 1 cytokines, particularly IFN–γ are predominant, but as the disease regresses type 2 cytokines become prominent and could be associated with lesion resolution.

Several lines of evidence indicate that HSK, a common cause of blindness in humans, is an immunopathological disease (Doymaz and Rouse, 1992). From work with mouse models of HSK, it is evident that T cells play an essential part in the pathogenesis (Metcalf et.al., 1979, Russell et.al., 1984 and Mercadal et.al., 1993), but exactly how T cells subserve an immunopathological role remains to be defined. Most evidences point to T cells of the CD4+ phenotype as major participants (Newell et.al., 1989a and 1989b, Hendricks and Tumpey, 1990, Niemialtowsky and Rouse, 1992 and Hendricks et.al., 1992,), but whether type 1 (Th1) or type 2 (Th2) cytokine-producing CD4+ T cell subset plays the principal role remains controversial. Previous works from two groups have advocated that CD4+ T cells producing type 1 cytokines are principally involved in the pathology (Niemialtowsky and Rouse, 1992 and Hendricks et.al., 1992). However, in apparent conflict with those findings, Jayaraman and colleagues (1993) have reported that HSV glycoprotein D (gD) specific T cell clones of Th2 characteristics can mediate HSK . In the earlier studiesin which Th1 CD4+ cells were advocated as pivotal in the pathological process (Niemilatowski and Rouse, 1992), the disease was only investigated at the clinical phase and ocular cells were secondarily activated *in vitro* with antigen or mitogen to induce cytokine production. In the present report, we have isolated RNA from the corneas and the DLN cells of mice infected with HSV at different stages of the disease process, and quantified cytokine mRNA levels present in such samples without any restimulation. Accordingly, the results would represent which cytokine genes were transcriptionally active at the time of sacrifice. The results suggest that the pattern of cytokine expression changes during different clinical phases of HSK. Early on, and until the time of peak clinical expression type 1 cytokines dominated the scene, but during

clinical resolution a reverse profile was evident with higher levels of type 2 cytokines (IL-4 and IL-10).

Although the data indicate the prominent role of type 1 cytokines during the preclinical and clinical phases of HSK, the cytokine mRNA most prominently detectable was IFN–γ Indeed, except at the peak clinical manifestation it was difficult to quantify mRNA for the type 1 cytokine IL-2. One likely explanation for the detection of abundant IFN–γ is that several cell types such as NK cells (Biron, 1994) and possibly gd T cells that comprise the inflammatory reaction could produce IFN–γ, whereas IL-2 is mainly a T cell product expressed by the few T cells in the inflammatory reaction. Alternatively, whereas IL-2 secretion by the corneal cellular infiltrates following *in vitro* stimulation could be demonstrated (Niemialtowsky and Rouse, 1992), the low levels of IL-2 mRNA expression *in vivo* may also indicate that the signal for the induction of IL-2 transcription might be lacking in the corneal milieu. This notion was recently documented by *in vitro* experiments on corneal endothelial cells (Kawashima et.al., 1994). To resolve the issue as to the type of cells producing IFN–γ and other cytokines during various clinical phases of HSK, will require the identification of cells in the cornea that produce various cytokines.

Infection with HSV, as with most other viruses, tends to induce predominantly a Th1 T cell response (Willie et.al., 1989, Gessner et.al., 1990, Huang et.al., 1993, Karupiah et.al, 1993, Nguyen et.al. 1994, Huber et.al., 1994, Sally and Pfaeffle, 1994,) and cells with this phenotype can mediate a delayed type hypersensitivity (DTH) reaction (Binachi et.al., 1981, Fong et.al., 1989 and Mosmann and Coffman, 1989, and Sarawar et.al., 1994). In most locations, the inflammatory reaction associated with such response is considered as protective. Indeed, IFN–γ dominated responses to HSV in the skin have been advocated as the principal means of recovery from HSV infection (Smith, et.al., 1994). However, in locations such as the cornea with HSV, or the brain as occurs in Theiler's virus infection (Clatch et.al., 1985 and Peterson et.al., 1993), or the heart as may occur in Coxsackie virus infection (Huber et.al., 1994 and Sally and Pfaeffle, 1994), the inflammatory response associated with a Th1 dominated reaction represents a major sacrifice in order to achieve viral clearance. In the case of the eye, for example, the response frequently results in blindness which persists long after virus has been cleared from corneal tissue. Interestingly, when infections such as HSV occur in certain locations such as the anterior chamber of the eye, an altered immune response

may occur which is characterized by a selective absence of a Th1 mediated DTH reaction (Streilein 1993). In fact, such an altered immune response to HSV had been shown to prevent HSK (Ksander and Hendricks, 1989) and further argues in favor of CD4+ T cells of DTH phenotype in the pathogenesis.

Although the data demonstrate a predominance of IFN–γ during the development and expression of clinical lesions, they do not resolve if IFN–γ is directly or indirectly involved in tissue damage. Some work by others on HSV pathogenesis does imply a mechanistic role for IFN–γ in pathology. Thus, animals develop less evident HSK lesions if additionally given anti IFN–γ, but a similar inhibition does not occur in infected mice suppressed with anti IL-4 or to the same extent with anti IL-2 (Hendricks et.al., 1992). Further evidence, which may implicate IFN–γ as mechanistically involved in herpetic ocular pathology, is the recent observation that the ectopic expression of IFN–γ in ocular tissues results in an increased inflammatory response following HSV infection (Geiger et.al., 1994). Perhaps arguing against a role for IFN–γ, either directly or indirectly, in mediating lesions is the observation that the adoptive transfer of a Th2 virus specific clone can exacerbate HSK (Jayaraman et.al., 1993. However, the recipient animals in this study were not immunocompromised and the contribution of CD4+ Th1 cells of the host origin was not excluded. Furthermore, the effect of Th2 clones in mediating HSK in the resistant mice was moderate and the lesions were resolved before the HSK lesions were manifested in the normally susceptible mouse strains. Clearly, it is important to unravel the exact role that IFN–γ plays in mediating directly or indirectly the ocular pathology.

Whereas type 1 cytokines predominated in the early and clinical phases of HSK, during the resolution phase type 2 cytokines were significantly expressed. Indeed although coexpression of low levels of IL-4 mRNA with IFN–γ were detected early on in the disease process in some corneal samples, IL-4 levels during remission were far higher. Moreover, only at the resolution phase was it possible to quantify IL-10 mRNA in both the corneal as well as in the DLN samples. IL-4, however, was expressed in the DLN cells throughout the course of the disease. It will be important to establish the reasons for the change in the cytokine profile in the cornea particularly in terms of the stimuli involved and the mechanism by which it is achieved. Accordingly, the change could represent either differential activation and/or recruitment. Of particular interest is the selective expression of IL-10 mRNA during the later phase of the HSK when

resolution was occuring. The stimulus for the recruitment and activation of IL-10 producers remains unknown. It is unlikely to represent infectious virus or recently synthesized viral products, since both are undetectable in the cornea long before the recovery phase (Metcalf and Kaufman, 1976, and Thomas and B. T. Rouse, unpublished). Despite the apparent absence of a viral stimulus, the corneal environment is the one where transcription activation of cytokine genes occurs at a far higher level than in the DLN cells at the same time.This may imply that some agonist, possibly another cytokine, an upregulated host protein or a persistently expressed viral antigen remains in the cornea for a prolonged period.

Whatever the mechanism responsible for triggering the shift to type 2 cytokine production, it seems likely that IL-10 itself could be involved either in inhibiting directly the proinflammatory effects of type 1 cytokine producing cells or through accessory cell dysfunction (Fiorentino et.al., 1989, Oswald et.al., 1992, D'Andrea et.al., 1993, Del Prete et.al., 1993, Enk et.al., 1993 and Kasama et.al., 1994). There is ample precedence for this notion in other well-studied systems where type 1 cells are involved in pathogenesis (Sieling et.al., 1993, Modlin and Nutman, 1993, Eckwalanga et.al., 1994, Ortwin-Rott et.al, 1994). Furthermore, the recent report of Tumpey and colleagues indicates a regulatory role for IL-10 in HSK (Tumpey et.al., 1994). Accordingly, infected animals developed diminished HSK lesions when IL-10 was given intrastromally as well as parenterally. Similarly, treatment of human HSK corneal specimens with IL-10 has been demonstrated to reduce the expression of class II MHC molecules that are known to be upregulated by IFN–γ in many cell types including the cornea (Boorstein et.al., 1994 and Abu El-Asar et.al., 1990). It will be instructive to establish if IL-10 given at later times in the experimental murine HSK can change the course of events, or alternatively if anti-IL 10 administration can abort the resolution process.

PART IV

Studies on the Induction and Effector Functions of HSV-1RE Immune and Naive CD4+ T cells in the Reconstituted SCID Mouse Models of HSK

1. ABSTRACT

In this section, both naive and HSV immune CD4+ T cells were transferred into SCID mice and manifestation of HSK was studied at both afferent and efferent levels. The results indicate that in both immune and naive CD4+ T cell reconstituted SCID mice, spontaneous cytokine mRNA expression in the pathological cornea was dominated towards Th1 cytokine profile, with expression of IFN-γ mRNA being frequently seen. The cytokine profile of the pathological cornea did not reflect the state of the donor cell cytokine pattern. The Th1 cytokine response of the CD4+ T cells in the cornea appear to be selective activation within the corneal milieu rather than selective homing of Th1 cytokine producers. Accordingly, although Th1 cytokine (IFN-γ) was secreted by the corneal inflammatory cells in response to mitogenic and HSV specific stimulation, various non secreted cytokine mRNAs could be induced following *in vitro* stimulation. In addition, very few T cells isolated from the pathological cornea secreted higher levels of IFN-γ in culture supernatant. Furthermore, even though HSV specific proliferative response was evident in peripheral lymphoid cells of naive CD4+ T cell reconstituted recipients, the inflammatory T cells isolated from HSK lesions were partially anergic to HSV specific proliferation, but responded to mitogenic stimulation. In contrast, in recipient spleen, the donor cells (both immune and naive) secreted higher levels of Th2 cytokine (IL-4) in response to HSV specific stimulation and IL-2 secretion was found to be minimal in relation to IL-4 levels. In addition, self reactive T cell responses were also evident in the T cells collected from peripheral lymphoid tissue of both euthymic as well as in reconstituted SCID mice, in response to HSV infection. Taken collectively the response of lymphocytes before and after adoptive transfer in terms of a) the cytokine secretion pattern and b) the *in vitro* proliferative responses, the data indicate that in the recipient environment the population of donor cells (immune or naive) that home in the cornea and in the peripheral lymphoid tissue (spleen) are different and / or behave differently at their effector site. In addition, the exacerbated response of HSK noticed in the reconstituted model is reflective of the activated state of the donor cell population.

2. INTRODUCTION

Following topical corneal infection with HSV-1 in euthymic BALB/c mice, clinical manifestation of HSK occurs in virus infected cornea but not in the contra lateral uninfected cornea. Although CD4+ T cells were involved in the pathogenesis of HSK (Doymaz and Rouse, 1992), with regard to the identity of CD4+ T cell effector subsets, namely Th1 or Th2, involved in HSK remain unresolved. Previous studies in euthymic animal models have implicated CD4+ Th1 cells in the pathogenesis (Niemialtowski and Rouse, 1992 and Hendricks et. al., 1992). As described in the previous chapter, *in vivo* expression of cytokine mRNA quantification during the course of HSK has further confirmed and added support to the notion that the disease is primarily mediated by CD4+ Th1 effector subset. Accordingly, early on and throughout the clinical manifestation of HSK Th1 cytokines were expressed in the cornea and Th2 cytokines were in fact expressed during the resolution of HSK. In contrast, in an adoptive transfer model, others (Jayaraman, et. al., 1993) reported that CD4+ Th2 cells were involved in the pathogenesis of HSK. However, the recipients of Th2 clones were not immunocompromised and it was not known whether the recipient Th1 cells contributed to the HSK lesions. On the other hand, the T cell deficient mice that fail to manifest HSK following corneal infection with HSV-1 alone (Metcalf et. al., 1979, Mercadal et. al., 1993 and Akova et. al., 1993) offer a more useful model to study the role of CD4+ T cells and their effector molecules in the pathogenesis of HSK.

In addition, it appears that adoptive transfer models of HSK differ in many respect from the euthymic mice. In reconstituted models HSK lesions were manifested in exacerbated fashion (Mercadal et. al., 1993 and Jayaraman et. al., 1993) unlike in euthymic mice. Furthermore, reconstitution of both immune and naive T cells into HSV-1 infected nude (Russell, et.al. 1984) and SCID (Mercadal et. al., 1993) mice resulted in manifestation of HSK. Thus it is not known whether the response of the naive CD4+ T cells to manifest HSK is due to HSV induced specific immunity in the recipient environment or due to other T cell recognition events.

The above mentioned differences in manifestation of HSK between euthymic BALB/c and reconstituted mouse models questioned, whether the pathogenesis of

HSK is mechanistically similar to that of natural course of lesion development in euthymic mice. Since in T cell deficient mice manifestation of HSK occurs only after CD4+ T cell reconstitution, the state of donor cell population before and after transfer into T cell deficient recipient mouse is readily definable and offers advantage to study the role of CD4+ T cells and its effector molecules in HSK. Thus the pathogenesis of HSK in reconstituted SCID mouse model was studied in two perspectives:

a) At the effector level with regard to cytokine production (efferent mechanism)

b) At the level of induction (afferent mechanism)

3. MATERIALS AND METHODS

3. 1. Mice

Four to five weeks old euthymic BALB / c, (Harlan Sprague Dawley, Inc. Indianapolis, IN) and CB-17($H2^d$) $^{SCID/SCID}$ (Taconic, German Town, N.Y.) were used for experiments. The animals were acclimated for 5 - 7 days before use in the experiments in specific pathogen free animal colony accredited by the American Association for Accreditation of Laboratory Animal Care. The SCID mice were housed in sterile microisolator cages. All food, water, bedding and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. All experimental procedures were conducted according to the association for research in vision and ophthalmology (ARVO) resolution on the use and care of laboratory animals.

3. 2. Corneal infection and clinical observation

The corneal surface of the deeply anesthetized mice (methoxyflurane [Metofane]; Pittman-Moore, Mondelein, Ill.) was scarified with a 27-guage needle and 5 x 10^6 50% tissue culture infective dose ($TCID_{50}$) HSV-1/ RE strain grown on Vero cells was applied in 4 µl volume and gently massaged with the eye lids. To increase the sample size animals were infected on both the corneas. Animals were examined at different days post infection by slit lamp biomicroscope (Keeler Instruments, Broomall, Pa.) and the severity of the stromal keratitis in each cornea was recorded with the following scoring system as described elsewhere for euthymic mice (Newell et. al., 1989a and 1989b): (0), clear cornea; (1^+), mild corneal haze; (2^+), moderate corneal opacity or scaring; (3^+), severe corneal opacity with visible iris; (4^+), opaque cornea, iris not visible; (5^+), necrotizing stromal keratitis.

3. 3. Donor cell preparation and adoptive transfer

Immune donor cells were isolated from the cervical and retropharyngeal draining lymph node of the euthymic BALB / C mice that were infected 7 days earlier with HSV-1 RE (5 x 10^6 $TCID_{50}$) on the cornea or from the spleen of i/p immunized mice. The naive donor cells were prepared from either the cervical and retropharyngeal lymph node or spleen. Single cell suspensions were made and RBCs

were lysed with ammonium chloride -Tris solution (pH 7.4) and the macrophages and dendritic cells were removed by adherence on plastic surface. The T cells in the non adherent cell population were enriched by depleting the B cells by two rounds of panning on the rabbit anti-mouse IgM coated plates. The resulting T cells were further enriched for $CD4^+$ T cells using the CD4 subset cloumn (R & D system, Minneapolis, MN) for adoptive transfer. The CD4+ T cell population prepared in this manner was transferred i/v into SCID mice that had been infected with HSV-1 RE on the cornea one day earlier. Control animals were infected with HSV-1 RE but were not reconstituted with T cells. A portion of the $CD4^+$ T cells used for adoptive transfer were used for flow cytometry and cytokine mRNA analysis.

3. 4. Isolation of corneal specimens for RNA and cell isolation

At different days post infection (p.i.) of the reconstituted SCID mice, the corneal pathology was scored as described earlier and the corneas were isolated at the termination of the experiment. The corneas were excised free of the scleral tissues and cut into fine pieces and used either directly for RNA isolation or used for preparing single cell suspensions . The single cell suspension of the corneal infiltrates were prepared by treating the cut corneal tissues for 45 min. at 37 ^{o} C in 1 ml of medium (10% fetal calf serum supplimented RPMI) containing 50 U of collagenase type I (Sigma) and 5 U of dispase (Boeringer Mannheim, Indianapolis, IN). Following digestion, the corneal samples were minced gently on fine stainless steel mesh. The dispersed cell aggregates were made in single cell suspension by gently plunging through a 21 gauge needle.

3. 5. Culturing corneal cellular infiltrates and preparation of fresh corneal cells from the reconstituted recipients

A single cell preparation was made from 16 corneas (clinical score $3^+/4^+$) of the naive splenic and lymph node $CD4^+$ T cell recipient SCID mice described earlier. 5×10^4 cells (~ 3×10^3 cells / cornea) were obtained. The cells were cultured in 48 well plate in the presence of naive X-irradiated (3500 rads) splenic feeder cells in 1 ml of limiting dilution medium (LDA) [1:1 vol/vol RPMI 1640 (GIBCO) and NCTC-109(Sigma), 10% heat inactivated fetal calf serum, 10mM L-glutamine (GIBCO), 1mM oxaloacetic acid(Sigma), 0.2 U/ml of bovine insulin (Sigma) and 5×10^{-5} M 2-

mercaptoethanol] containing 2 Units of rIL-2 / ml at 37° C for 5 days. Viable cells were isolated by passing the cells over Histopaque -1083(Sigma). A total of 10^6 cells were obtained and split cultures were used for proliferation and cytokine production. For fresh corneal cell isolation, 14 corneas were pooled from 6 x 10^6 naive spleen and lymph node $CD4^+$ T cell recipients. Total yield was 6 X 10^4 cells (~ 4 x 10^3 cells / cornea).

3. 6. Lymphocyte proliferation

Splenic single cell suspensions of the reconstituted recipients were lysed of RBCs and allowed to adhere to a plastic surface. The non adherent cell population was collected after a second round of adherence and the cells were added to X-irradiated (3500 rads) mock or 2 min. ultra violet (UV) inactivated HSV-1 RE (multiplicity of infection 5 prior to UV inactivation) infected (4 hr) syngeneic BALB/c splenic stimulator cells at various responder to stimulator ratio in triplicate in 96 well U-bottom plate containing 200 µl total volume of supplemented LDA. After 3 days in culture at 37° C, cells were pulsed with 1 µCi of [^{3}H]-thymidine for 12 hr and the levels of [^{3}H]-thymidine incorporation was determined in dry β-counter (INOTECH-96, Biosystems International, MI). In some experiments, two 50 µl of the culture supernatant was collected per well onto replica plates prior to adding [^{3}H]-thymidine. Similarly, proliferative analysis of the purified immune and naive donor $CD4^+$ T cells were performed on the day of adoptive transfer. For proliferative analysis of the cultured inflammatory corneal cell isolates, 10^4 cells / well were either stimulated with mock infected, HSV-1 RE infected X-irradiated syngeneic stimulator cells (5 X 10^4 cells/well), or with Con A (3 µg/ml) in triplicate cultures in U-bottom plate and the proliferation was assayed as described earlier.

3. 7. Stimulation of the corneal cells for cytokine induction

Freshly prepared (1.5 X 10^4) or cultured corneal cells (2 X 10^5) of the inflammatory cornea prepared as decribed above were stimulated with 10^6 X-irradiated syngeneic BALB/c splenic stimulator cells either uninfected or infected with HSV-RE or stimulated with Con A (3 µg/ml) for 72 hrs in 48 well plates containing 1 ml of LDA. The culture supernatants were aliquoted and kept frozen at -

80° C. At the end of *in vitro* culture, the cells were harvested and used for RNA isolation.

3. 8. Measurement of cytokine secretion in the culture supernatant

CTLL-2 (ATCC; TIB 214) and CT4.S cells (supplied by Dr. W.E. Paul, National Institute of Allergy and infectious Diseases, National Institutes of Health, Bethesda, MD) were used (5 X10^3 cells/well) for the detection of IL-2 and IL-4, respectively in the culture supernatant. Anti IL 4 antibody (7 μg / ml, Pharmingen) was added to the CTLL-2 cells to block the IL-4 dependent response. Cytokine dependent growth was assayed in triplicate in 96 well U-bottom plates. After 36 hrs of incubation, the cells were pulsed with [^{3}H]-thymidine for 12 hrs, and the measurement of growth was determined as described earlier. Recombinant IL-2 or IL-4 was used to generate the standard curve. Sandwich ELISA was used for the detection of IFN-γ (Gibco, BRL), and IL-10 (Endogen).

3. 9. Flow cytometry

A portion of the immune or naive donor T cells or the non adherent splenic cell suspension of the reconstituted recipient were analysed in two color staining in FACscan (Becton-Dickinson, Mountain View, CA) flow cytometer. Briefly, the cells were washed with Hank's balanced salt solution (HBSS) containing 1% BSA and 1% FCS and 0.5-1 x 10^6 total cells were stained in combination of either fluorescein isothiocyanate (FITC) or phycoerytherin (PE) conjugated antimouse CD4, CD8, CD45R, PgP-1, LFA-1, or Mel-14 for 1 hr. at 4°C. Isotype matched FITC or PE-conjugated antibodies were used as a control. Following staining, the cells were washed with HBSS containing 1% BSA and 0.2% sodium azide and fixed with 0.3% parafarmaldehyde in HBSS. The splenic cells of the recipients were stained with antibodies against αβ TCR. Analysis were done 24 hrs after staining in 5000-10000 events either in Consort 30 or Lysis (Becton-Dickinson) software. All antibodies used in the flow cytometry were obtained from Pharmingen, San Diego, CA.

3. 10. RNA preparation and quantitative polymerase chain reaction

RNA isolation, qualitative and quantitative PCR were performed as described previously (part III.). For semi-quantitative PCR analysis, the relative intensity of the

amplified products were calculated by imaging densitometer (BIORAD, model GS-670). The overall experimental design used in this section is illustrated in fig. 1.

Design of the adoptive transfer experiments

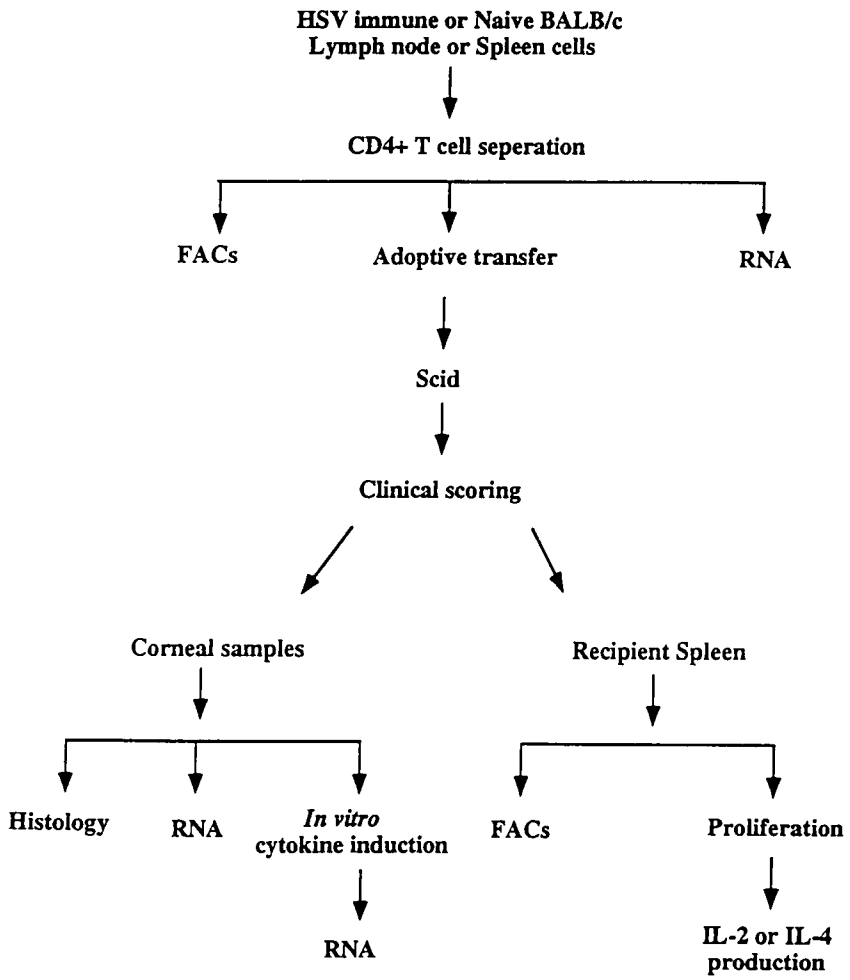


Fig.1.

Experimental design of reconstituted SCID mouse models of HSK.

4. RESULTS

4. 1. Early manifestation of HSK lesions in the immune CD4+ T lymphocyte reconstituted SCID mice

In certain ocular immunopathological conditions such as HSV induced retinitis, immune T cells primed *via* different routes behaved differently in their effector functions following reconstitution (Wittum-Hudson et. al., 1985). In order to see whether the response of immune $CD4^+$ T cells to induce HSK is influenced by the route of primary immunization, HSV immune $CD4^+$ T lymphocytes were purified from either the DLN or spleen of the donor BALB/c mice that were either infected on the cornea or by i/p route, respectively. As illustrated in fig. 2 and summarized in table 1, the route of primary immunization did not exert any influence on the effector $CD4^+$ T cells and both populations mediated the disease with same magnitude. However, unlike euthymic BALB/c mice, in the reconstituted SCID mice, the onset of the disease is earlier with severe clinical lesions. Furthermore the incidence of the disease was higher (80-100%) in reconstituted mice (fig.2 , table 1) compared to the euthymic mice in which 20~25% remain as non responders. In addition, in reconstituted SCID mouse models, resolution of clinical lesions were not evident and lesions progressed to severity at termination of experiment.

4. 2. Response of the lymph node and splenic naive CD4+ T cells in the manifestation of HSK

In earlier studies (Mercadal et. al., 1993) naive $CD4^+$T lymphocytes isolated from spleen were shown to induce HSK lesions similar to that of immune $CD4^+$ T cells. To further understand the role of naive $CD4^+$ T cells in inducing HSK lesions, $CD4^+$ T cells were collected from naive BALB/c lymph nodes and their ability to induce lesions was compared with splenic naive $CD4^+$ T cells. As illustrated in fig. 3A, the splenic $CD4^+$ T cell recipients manifested the disease earlier compared to the lymph node cell recipients. Similarly the incidence and severity of the disease was also higher in the splenic $CD4^+$ T cell recipients compared to the lymph node $CD4^+$ T cell reconstituted mice (fig. 3B). Such a response between the two naive $CD4^+$T cell population was considered at least in part due to the presence of activated T cells

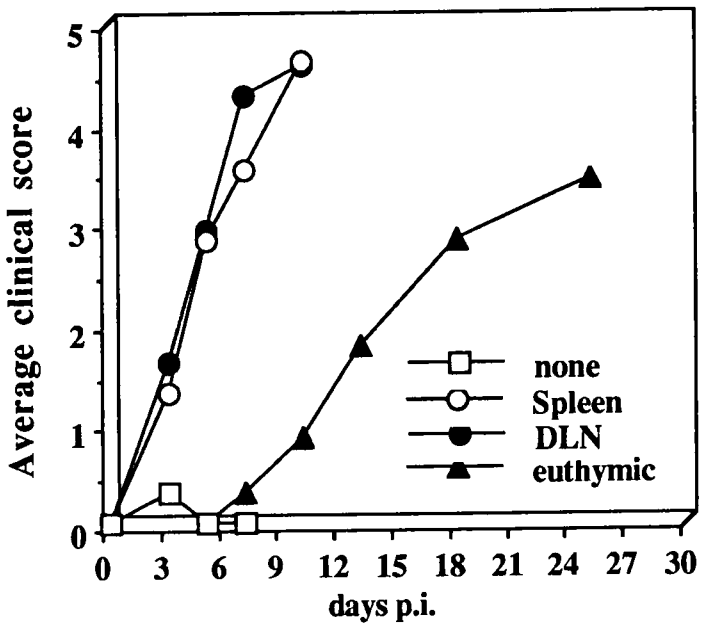


Fig.2.

Dynamics of HSK development in immune CD4+ T cell reconstituted SCID mouse models and in euthymic BALB/c mice.

CB-17 ($H2^d$) SCID mice were reconstituted with either 4 x 10^6 HSV-1RE immune splenic or lymph node CD4+ T cells by i/v transfer. The recipients were infected with HSV-1RE a day prior to transfer on both the corneas to increase the sample size. The manifestation of HSK in euthymic BALB/c mice in an unrelated experiment is shown. Number of animals used in the experiment: non reconstituted but HSV-1RE infected SCID, n = 5; immune CD4+ T cell recipients, splenic cells n = 4, DLN cells n = 6; euthymic, n = 20.

Table 1

Summary of immune splenic and lymph node CD4+ T cell transfer in reconstituted SCID mice.

Donor cell source [a]	Number of cells transferred X 10^6	% CD4+ cells [b]	Number of corneas with HSK [c]	Number of corneas with necrotizing HSK[d]
	none	---	0 / 10	0
Spleen	15	78	8 / 8	5
DLN	4	79	12 / 12	6

[a] Immune donor CD4+ T cells were prepared from the spleen of BALB/c mice immunized i/p or from the cervical and retropharyngeal lymphnodes of 23 mice infected on the cornea seven days earlier.

[b] CD4+ T cells were purified as described in the methods and portion of the donor cell population used for reconstitution was stained for CD4 and CD8 PE or FITC conjugated antibodies on the day of transfer and analysed 24 hrs later. The percentages of cells stained positive for CD4 is calculated in retrospect and summarized. Staining for CD8+ T cells were < 1% (data not shown) similar to that of the autofluorescence. For purity of the donor cell population refer to Fig. 4.

[c] The recipient animals that manifested the clinical score of 3+ and above at the termination of experiment at 11 d post transfer (12 d p.i.).

[d] Number of corneas manifested necrotizing HSK lesions at termination of the experiment. Part of the data is illustrated in Fig. 2.

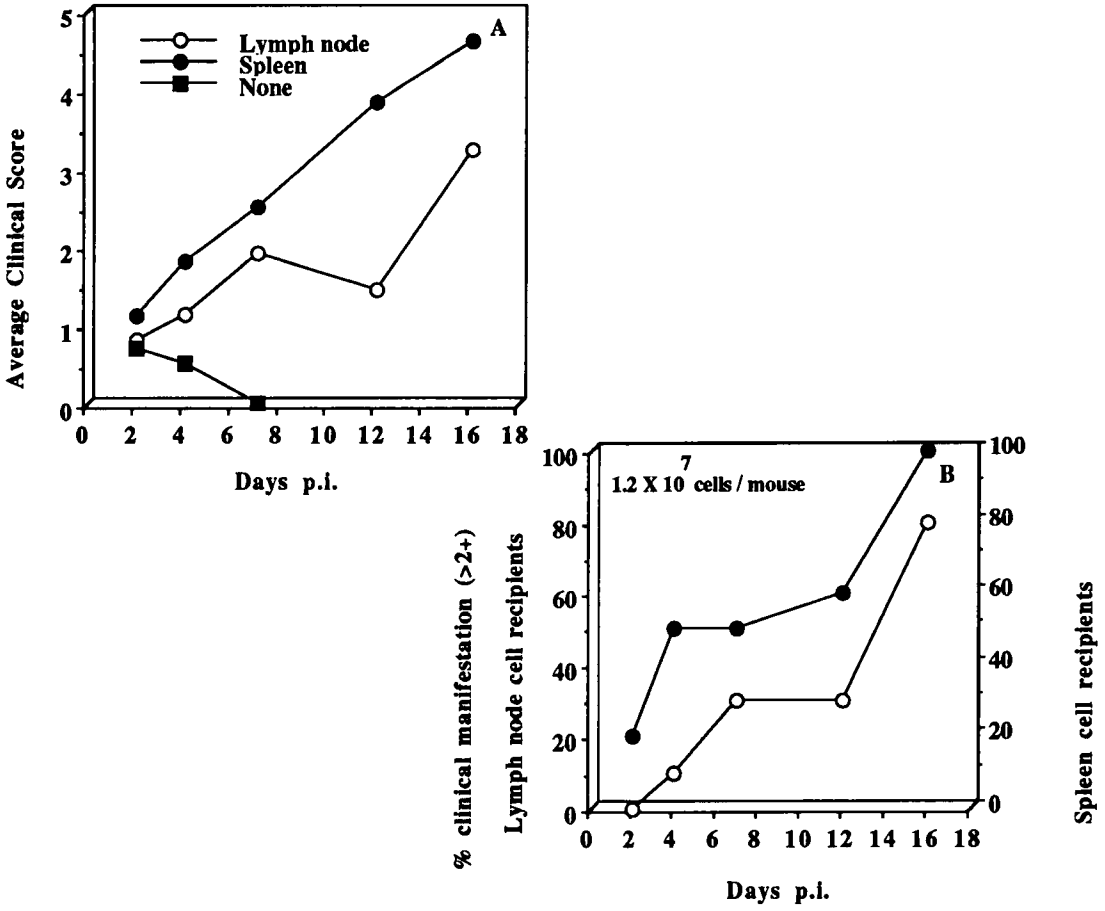


Fig. 3.

A) Manifestation of HSK in the naive splenic and lymph node CD4+ T cell recipients and B) percentages of animals manifested the lesions.

CD4+ T cells were purified from 25 naive BALB/c mice kept under specific pathogen free environment and 1.2 x 10^7 cells / mouse (spleen cell recipients n = 8; lymph node cell recipients n = 6) were transferred i/v to the HSV-1RE infected SCID mice. Three SCID mice were infected with HSV-1 but were not reconstituted. Animals were infected on both the eyes to increase the sample size. Experiments were terminated when necrotizing lesions were evident in some corneas in both the groups. Corneas that showed clinical score of 3/4+ were pooled from both the groups for isolation of cells for cytokine assay. The purity and the characteristics of the donor cell population is shown in Fig. 4

in the donor cell population. Accordingly, as illustrated in fig. 4, ~ 23 % of the splenic donor cells exhibited the activated phenotype as evident by the Mel-14 $^{-}$, and CD45RB ^{low} and PgP1 ^{high} and LFA-1^{high} expression (Budd et. al., 1987 and Lee and Vitetta, 1991) compared to the lymph node cell population where only 6% of the cells were found to be of activated phenotype. Similar findings were also noticed, when half the cell dose of naive CD4^{+} T cells were used for reconstitution (table2). However, unlike the immune CD4^{+} T cell recipients, the manifestation of HSK lesions were delayed by a few days in the naive cell recipients (Fig. 3). In one experiment, however, when naive CD4^{+} splenic cells that contained a higher proportion of activated T cells were used, severe HSK lesions were manifested by 12 day post transfer (table 2).

Although the time period at which HSK lesions were manifested varied depending upon the donor cell phenotype, histologically the clinical lesions were indistinguishable between the immune(fig. 5A) and naive (fig. 5B) cell recipients and were similar to that of the euthymic mice(fig. 5C). Furthermore, in non reconstituted SCID mice, infection with HSV resulted in minimal damage to the cornea examined at 7 day p.i. (fig. 5D). In addition to manifesting HSK, as evident in fig.2 and 3, protection from viral encephalitis and subsequent mortality was also noticed in both the immune and naive CD4^{+} T cell reconstituted SCID mice compared to the non reconstituted virus infected ones. However, when low numbers (<5 X 10^{6}) of cells were used for reconstitution, some mice did succumb to encephalitis but mortality was delayed.

4. 3. Spontaneous expression of IFN-γ mRNA in the inflammatory cornea

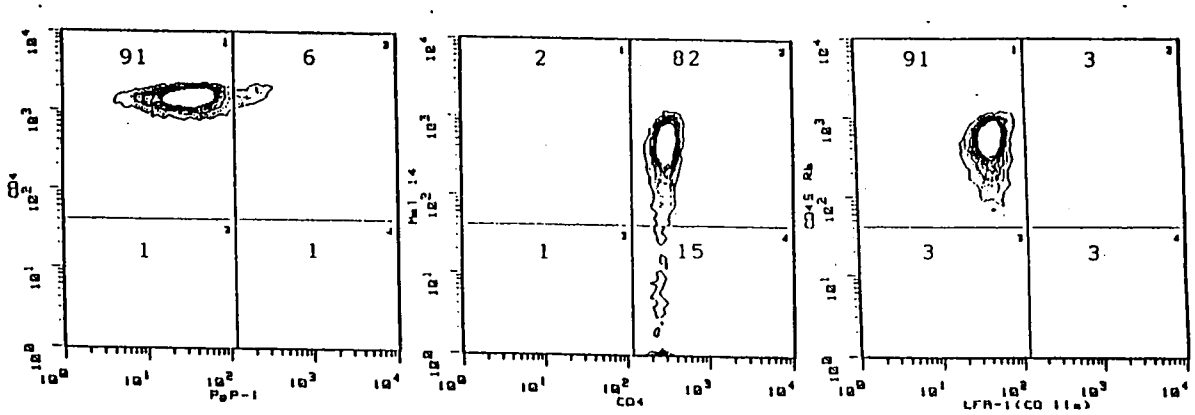
In order to determine the cytokine profile in the inflammatory cornea in the reconstitution models, the IL-2, IL-4, IL-5, IL-10 and IFN-γ mRNA expression was analysed by qualitative RT-PCR and quantified (10-12 days post transfer) . In both naive and immune CD4^{+} T cell reconstituted SCID mice preliminary experimental samples, frequently expression of IFN-γ but not other cytokine mRNA was evident. However, in an experiment when 1.5 X 10^{7} immune CD4^{+} T cells were used for reconstitution, a weak coexpression of IL-2 and IL-4 but not IL-5 or IL-10 mRNA

Fig.4.

Flow cytometric analysis of the naive CD4+ donor T cells.

CD4+ T cells from cervical, retropharygeal lymph nodes and spleens of naive BALB/c mice were prepared as described and used as donor cells for reconstitution of SCID mice (Fig.4) Staining for naive and activated cell phenotypes was done in a portion of the donor cell population prepared from the lymph node (top) and spleen (bottom). Cell were stained on the day of transfer and 10^4 events were analysed 24 hrs later. The percentages of cells were shown in each quadrant.

Lymph Node



Spleen

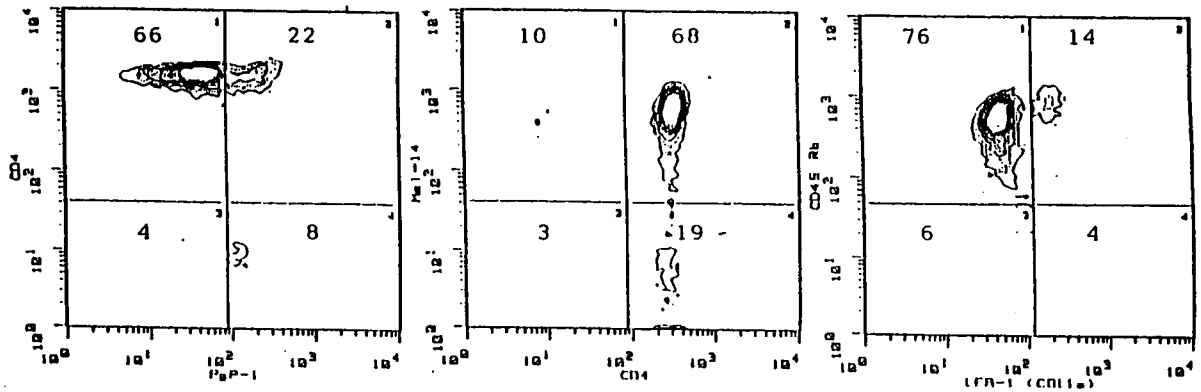


Table 2

Summary of naive splenic and lymph node CD4+ T cell reconstitution in SCID mice

Donor cell source [a]	Number of cells transferred X 10^6	% CD4+ cells [b]	Number of corneas with HSK[c]	Number of corneas with necrotizing HSK[d]
Spleen	6	81	6 / 6	4
	6	73	10 / 14	2
	12	85	8 / 10	5 / 10
Lymph node	6	92	12 / 16	2 / 16
	12	89	10 / 12	2 / 16

[a] The recipients were infected with HSV-1RE a day prior to transfer and reconstituted i/v with naive CD4+ T cells prepared from spleen or lymph nodes of 25 naive BALB/c mice.

[b] The purity of the donor cell population was analysed in retrospect by CD4 and CD8 staining and the percentages of cells stained positive for CD4 is summarized. The purity of the donor cell population and their characteristics is illustrated in Fig. 5.

[c] Number of corneas that manifested a clinical score of 3+ and above at the termination of experiment.

[d] Number of corneas that manifested necrotizing lesions at the termination of the experiment. Experiments were terminated after 14-16 d p.i. for collection of viable cells from the cornea and for RNA isolation. Part of the data is illustrated in Fig. 3

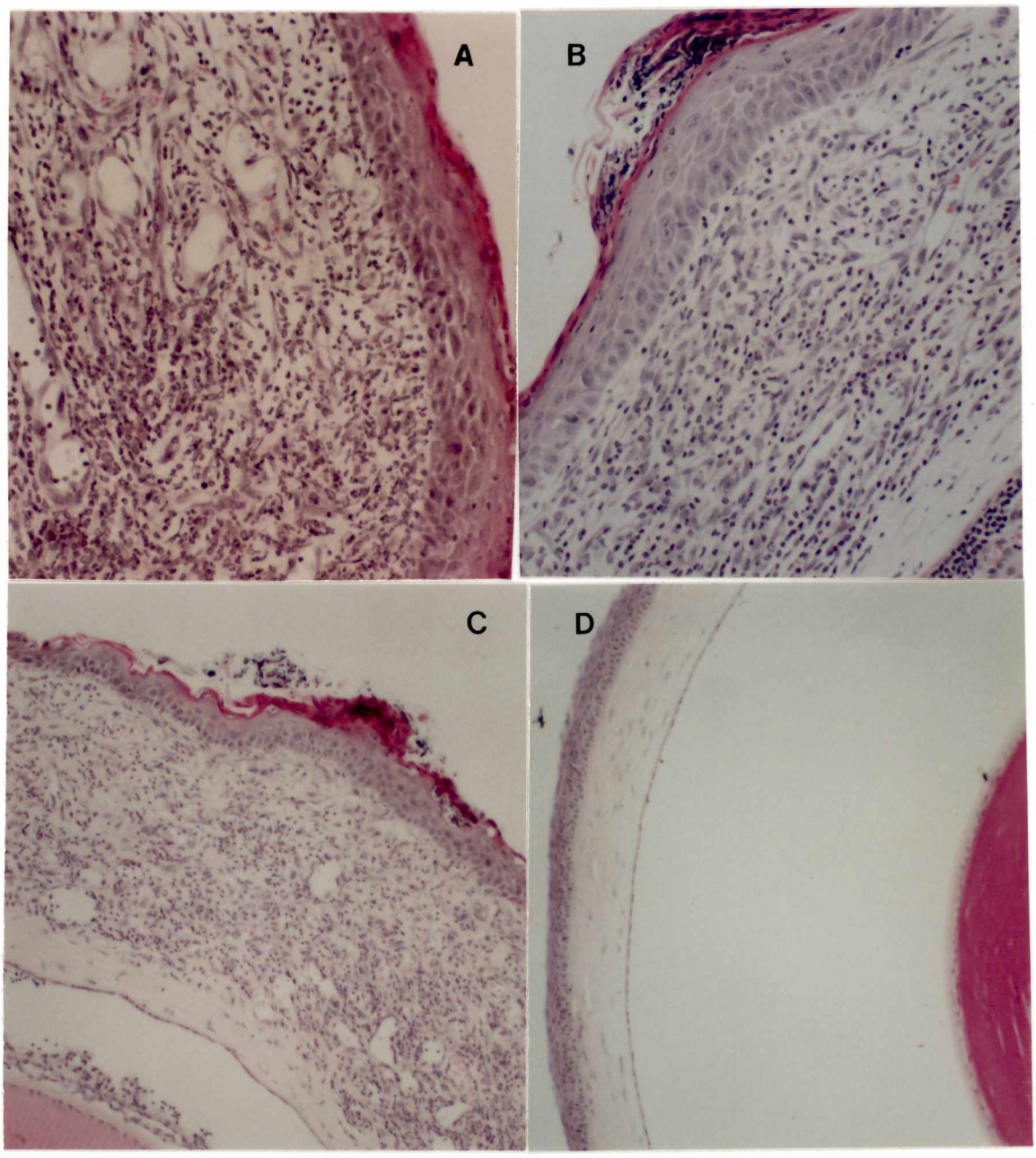


Fig. 5

Histological sections of immune and naive CD4+ T cell reconstituted and non reconstituted SCID mice cornea

A) immune, B) naive CD4+ T cell recipient SCID mice, C) euthymic mice, and D) non reconstituted but HSV-1RE infected SCID mice. Original magnification 20X.

expression were evident along with IFN-γ (table 3). Although none of the cytokine mRNAs analysed in this study were expressed in the naive SCID cornea, in the non-reconstituted but virus infected SCID corneas examined at 7 day p.i., weak expression of IFN-γ mRNA was evident. However, at quantitative levels, the IFN-γ mRNA copies were found to be ~ 300 fold lower compared to the inflammatory cornea. A representative result of corneal cytokine mRNA levels in immune $CD4^+$ T cell recipients is summarized in table 3. It is interesting to note that although in the immune splenic donor cells both Th1 and Th2 cytokine mRNA were expressed (table 4), the spontaneous cytokine mRNA expression in the cornea is confined mainly towards Th1 type cytokines. However, although neither IL-2, IL-4, IL-5, nor IL-10 mRNA expression was evident in both naive splenic and lymph node donor $CD4^+$ T cells, low levels of spontaneous expression of IFN-γ (~ 1000 copies / 10^6 cells) mRNA was observed. Such spontaneous expression of IFN-γ mRNA in the naive cell population was also observed by others (Henderson et. al., 1992) and in earlier studies (Part II and III).

4. 4. *In vitro* secretion of IFN-γ but not IL-2, IL-4, and IL-10 cytokines from the corneal cellular infiltrates in response to HSV specific stimulation

In order to see if the spontaneous dominant expression of IFN-γ mRNA seen in the inflammatory cornea is due to selective homing of IFN-γ producing effector $CD4^+$ T cell type or if other cell types also home in the cornea but remain quiescent, fresh corneal cellular infiltrates were isolated from the naive $CD4^+$ T cell reconstituted SCID mice and stimulated *in vitro* with T cell mitogen or HSV-1 infected or uninfected stimulator cells (table 5 A). Interestingly enough, secretion of only IFN-γ but not other cytokines (IL-2, IL-4, and IL-10) was evident even after polyclonal stimulation with the mitogen. Such was the case when the cells were stimulated with HSV-1 infected stimulator cells. However, when the corneal cells used for *in vitro* stimulation were collected at the end of the culture period (72 hrs) and analysed by RT-PCR, induction of IL-2, IL-4, and IL-10 but not IL-5 mRNA was evident along with IFN-γ (fig. 6A), even though in the culture supernatant secretion of only IFN-γ but not other cytokines was evident. By semi-quantitative RT-PCR analysis, except IL-10 mRNA, the non secreted cytokine mRNA transcripts such as IL-2, and IL-4 did not differ much from that of IFN-γ that was secreted (fig. 6B).

Table 3

Expression levels of cytokine mRNA in immune CD4+ T cell reconstituted SCID mice cornea
(copies X 10^3) / cornea

mRNA species	Non reconstituted HSV infected	4 X 10^6 CD4+ T cell recipient	1.5 X 10^7 CD4+ T cell recipient
IL-2	NE	NE	<0.005*
IL-4	NE	NE	<0.005*
IL-5	NE	NE	NE
IL-10	NE	NE	NE
IFN-γ	7.6	1018.3	2099.2

Total cellular RNA was isolated fom 3 corneas of immune CD4+ T cell reconstituted SCID mice and from 6 corneas of non reconstituted but HSV-1RE infected SCID mice. The IFN-γ mRNA that was spontaneously expressed in high levels was quantified by competitive RT-PCR approach .

*weak expression was evident in qualitative RT-PCR, but fell below quantifiable limit. NE, not expressed

Table 4

Expression levels of cytokine mRNAs in HSV immune CD4+ splenic cells used for reconstitution

(mRNA copies X 10^5) / million cells

mRNA species	copy numbers
IL-2	465
IL-4	49
IL-5	3
IL-10	0.1
IFN-γ	1356

Total cellular RNA was isolated from immune splenic CD4+ donor cell population and the spontaneous expression of cytokine mRNAs were quantified by competitive RT-PCR approach.

Table 5A

In vitro cytokine secretion of fresh inflammatory corneal cellular infiltrates of reconstituted SCID mice (units / ml)

Cytokines	Naive stimulators	HSV-1 RE infected stimulators	Con A
IL-2	--	--	--
IL-4	--	--	--
IL-10	--	--	--
IFN-γ	--	6.6 +/- 2	27.5 +/- 6

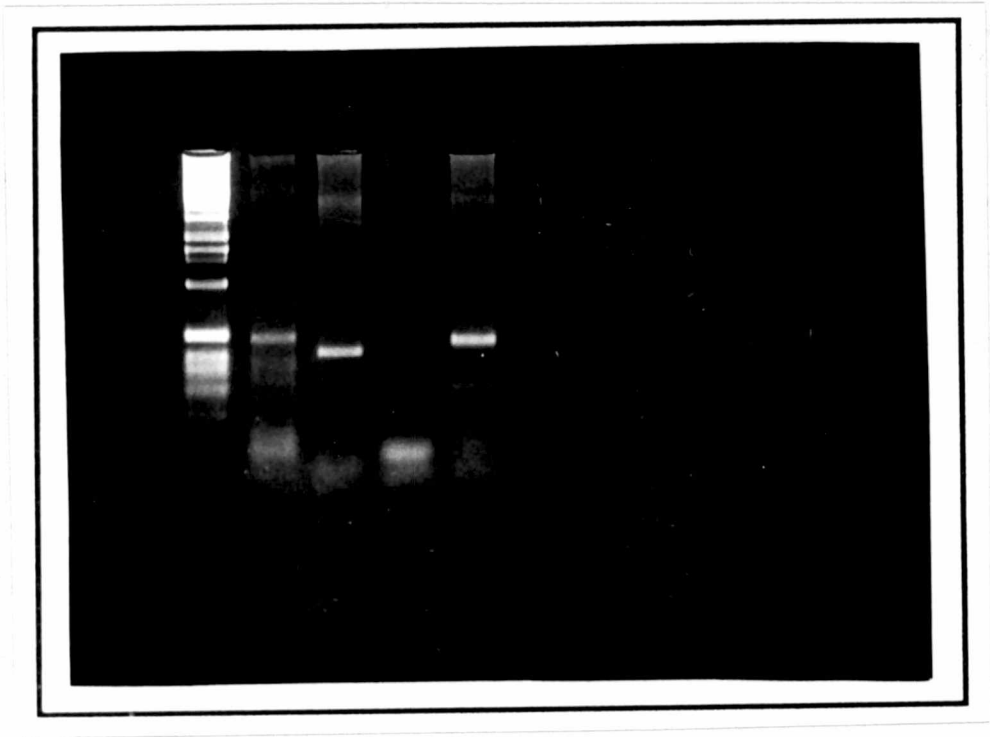
Table 5B

In vitro cytokine secretion of cultured inflammatory corneal cellular infiltrates of reconstituted SCID mice (units / ml)

Cytokines	Naive stimulators	HSV-1 RE infected Stimulators	Con A	Sensitivity and type of assay
IL-2	--	--	1.4 +/- 0.3	<0.2 (Bioassay)
IL-4	--	--	--	<0.3 (Bioassay)
IL-10	--	--	--	<1.0 (ELISA)
IFN-γ	--	53.1 +/- 17	33. 2 +/- 5	< 0.3 (ELISA)

1.5×10^4 freshly prepared corneal cells (Table 5 A) or 2×10^5 cultured corneal cells (Table 5B) were stimulated with uninfected or HSV-1RE infected syngeneic X-irradiated stimulator cells or with Con A (3 μg / ml) in 24 well plates in 1 ml of LDA medium for 72 hrs and assayed for cytokine secretion in the culture supernatant. Units of IL-2, IL-4 , and IFN-γ are defined by NIH standards. For IL-10, the least sensitivity of the ELISA kit was described as an unit. One unit corresponds to 100 pg for rIL-2 and 150 pg of rIL-4, 175 pg of rIFN-γ and 100 pg of rIL-10.

A



B

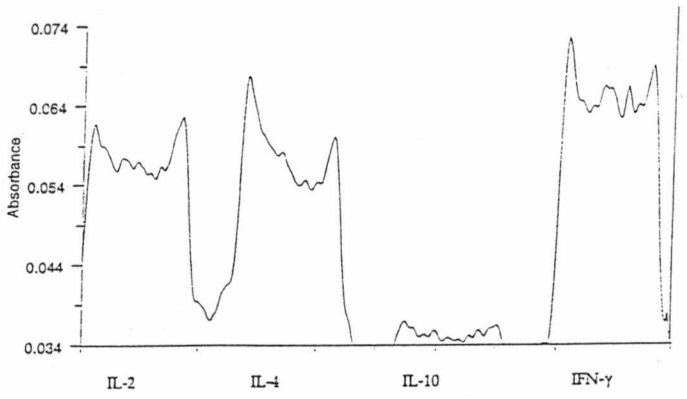


Fig.6.

Qualitative and semiquantitative RT-PCR analysis of *in vitro* stimulated corneal inflammatory cells analysed for cytokine mRNA expression at the end of *in vitro* stimulation (table 5A).

A) Lane 1: I Kb Ladder, Lane 2: IL-2, Lane 3: IL-4, Lane 4: IL-10, and Lane 5: IFN-γ), B) Profile of the relative levels of cytokine mRNA expression analysed by semi-quantitative RT-PCR

These results indicate that although (except IL-10) the IL-2, IL-4 and IFN-γ producers were present at near equal amounts, only the IFN-γ producers actively secrete the cytokine.

On the other hand, when the inflammatory corneal T cells of naive $CD4^+$ T cell reconstituted SCID mice were cultured *in vitro* prior to stimulation, in addition to IFN-γ, secretion of IL-2 was evident (table 5B). Additional induction of IL-2 was evident only upon T cell mitogenic stimulation but was not evident with HSV specific stimulation, although IFN-γ was secreted in response to virus specific stimulation. Furthermore, it is interesting to note that the levels of IFN-γ secreted after mitogenic stimulation did not differ quantitatively from that of HSV induced stimulation. Only additional induction of IL-2 was evident. Such a response indicates that antigen specific stimulation selectively activates IFN-γ producers, while mitogenic stimulation results in coactivation of IL-2 producers along with IFN-γ producers.

4. 5. Corneal T cells that secreted IFN-γ were partially anergic to HSV specific proliferative response but not to mitogen

When split cultures of the cultured corneal inflammatory T cells that were used for *in vitro* cytokine induction (table 5B) were assessed for HSV-1RE specific proliferative response, the cells were anergic to virus specific stimulation but readily proliferated in response to mitogenic stimulation (Fig.7). The lack of HSV specific proliferative response was considered not due to purification procedures, since HSV immune T cells treated in the same manner did respond to HSV infected stimulator cells. Proliferative and cytokine response of the corneal cellular infiltrates to T cell mitogen indicated the presence of the donor T cells, since resident SCID cells are refractory to mitogen .

4. 6. Self reactivity of the donor cell population in the reconstituted SCID spleen

In order to gain insight into the fate of the adoptively transferred naive and immune $CD4^+$ T cells, splenic cells were isolated from the reconstituted SCID mice as described in the methods at the termination of experiments (12-16 days post transfer) . The reconstituted SCID splenic cell isolates were blasts and 70-83% of the cells were αβ TCR positive as evaluated by flowcytometry. However, in 14 mice analysed, in absolute cell number the total recovered donor lymphocytes constituted

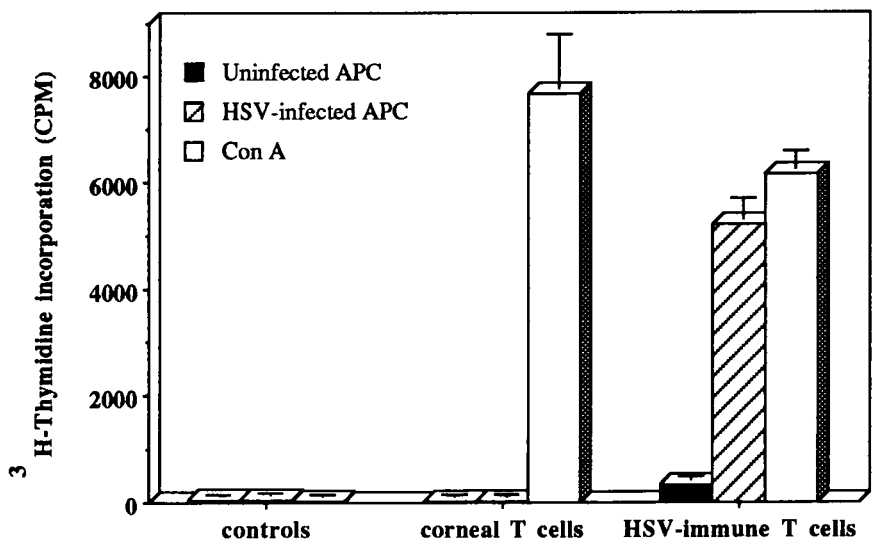


Fig.7

Lack of HSV specific proliferative response in the HSK corneal cells.

Single cell suspensions of the 16 corneas of 1.2 X 10^7 naive spleen and lymph node CD4+ T cell recipent SCID mice (table 2, Fig.3) were prepared and cultured *in vitro* as decribed in the methods. 10^4 cells of the split cultures of the cultured corneal cells that were used for cytokine production (table 5B) were either stimulated with or without HSV-1RE infected syngeneic BALB/c stimulator cells in 96 well U-bottom plates in triplicate culture or stimulated with Con A (3μg /ml). HSV immune T cells (7d p.i.) of the BALB/c mice that were subjected to collagenase-dispase digestion and *in vitro* culture as that of the corneal cells were used as positive control.

only 10-25 % of the input cell number. However, when the recipient splenic cells were analysed for HSV specific proliferative responses, surprisingly, high degree of self reactivity was evident in both both immune and naive $CD4^+$ T cell recipients. The representative results are summarized in table 6. The self reactive T cells were noticeable only in the reconstituted virus infected mice but not in the uninfected but reconstituted recipients. The response to HSV antigens were often undefinable due to strong auto proliferative response. However, when the responses were analysed in individual mice, HSV specific response could be demonstrated over auto proliferative responses, although to a minimal level. (fig.8) In addition, the self reactive response was also seen between the T cell population, since even in the absence of any syngeneic stimulator cells, auto proliferative response was evident (table 6, fig.8).Such an effect could be mediated by a few contaminating antigen presenting cells (APC) of SCID origin. However, the influence of contaminating APC was considered to be very minimal because >70% of the responder population contained T cells and in absolute cell number the possible contaminating APC was considered very few. On the other hand, the auto proliferative response could be due to the blast nature of the responder cells that are known to grow independent of stimulator cells in short term culture. Nevetheless, self reactive responses were also evident in HSV immune BALB/c donor $CD4^+$ T cells in response to syngeneic stimulator cells (Fig.9).

4. 7. Differential cytokine response in the recipient splenic cells

Because both the immune and naive donor T cells demonstrated self reactivity in HSV infected recipient spleens (table6, Fig. 8), the cytokine response of the donor cells isolated from the recipients was determined. In this regard, splenic cells of three naive and two immune T cell recipients were analyzed individually and the cytokine response to self and HSV-1 was determined. The results of two mice in which HSV specific response(SCID 1, naive cell recipient) or strong auto reactivity (SCID 2, immune cell recipient) was evident are illustrated in fig.10 A. When the culture supernatant was analysed in replica plates, secretion of IL-2 was either not evident or found to be at a very minimal levels (fig. 10B) . However, high levels of IL-4 secretion was evident in the culture (fig. 10C). Furthermore, the levels of IL-4 in response to HSV specific stimulation (fig.10C) was higher in the responder

Table 6.

Pooled splenic cells of 3 mice per group were prepared as described in the methods and 2.5 x 10^4 responders were stimulated with HSV-1RE infected or uninfected X-irradiated BALB/c stimulator cells in triplicate cultures. One μCi of ^{3}[H]-thymidine was added to each well after 3 days in culture and cells were harvested 12 hr later. The ^{3}[H] thymidine incorporation was counted by Trace-96 INOTECH β-dry counter. The phenotypes of the cells were > 70% αβ TCR+. HSV-1RE infected SCID mice were reconstituted with either 4 X 10^6 immune CD4+ T cells (table 1) or 6 x 10^6 naive CD4+ T cells (table 2). Uninfected but reconstituted SCID mice recieved 5.5 x 10^6 naive CD4+ T cells. Standard deviation of the culture < 10%.

Table 6

Self reactive splenic T cells in HSV-1RE infected reconstituted SCID mice
(^{3}H-thymidine incorporation [Average CPM]

Groups	Media	Stimulators alone	Responders alone	Responders + naive stimulators	Responders + HSV infected stimulators
Naive	107	23	70	88	43
HSV infected non reconstituted	46	17	42	60	53
Naive reconstituted	22	27	77	67	97
HSV infected + Naive CD4+ T cell reconstituted	170	29	27329	33852	13395
HSV infected + Immune CD4+ T cell reconstituted	70	42	5582	8478	14903

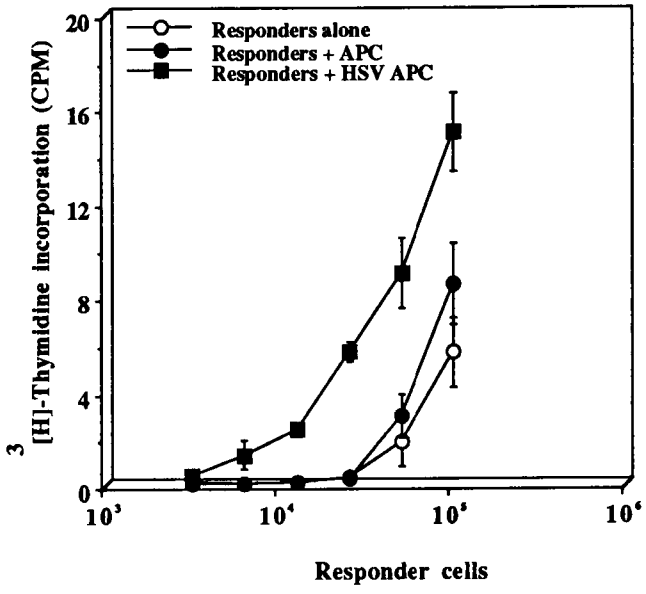


Fig. 8

A representative self reactive and HSV specific responses in the reconstituted SCID mouse spleen cells.

HSV-1RE infected 4 SCID mice that had recieved naive 6 x 10^6 CD4+ T cells from the donor BALB/c mice collected at the termination of the experiment (15d post transfer). The responder cells were prepared from the non adherent cell population of recipient spleen as described in the methods and culture in triplicate in 96-well U bottom plates in varying responder cell numbers. The cells were either unstimulated or stimulated with 10^5 X-irradiated syngeneic BALB/c splenic stimulator cells that were either uninfected or infected with HSV-1RE. Cells were pulsed with 1 μCi of [^{3}H]-thymidine for 12 hrs after 3 days in culture. A portion of the responder cells used in this assay from each mouse were stained for αβ TCR expression on the day of assay and analysed by flow cytometry 24 hrs later. 69 % of the illustrated responders contained αβ TCR + T cells.

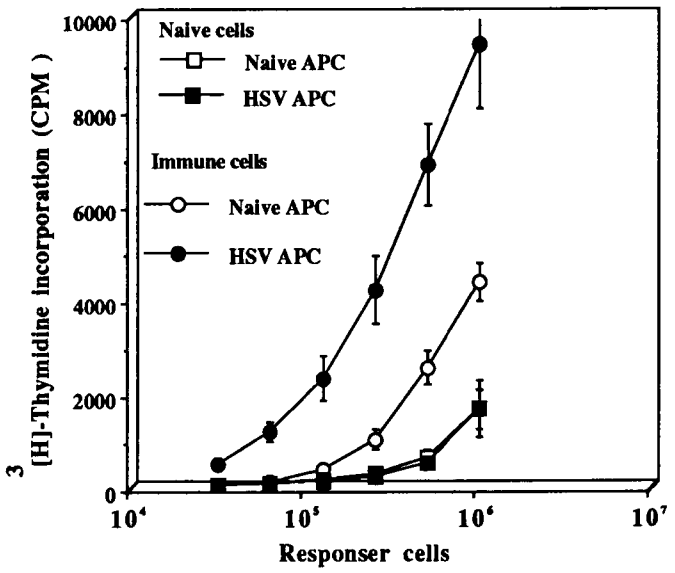


Fig.9

Self reactive responses in HSV immune T cells of BALB/c mice

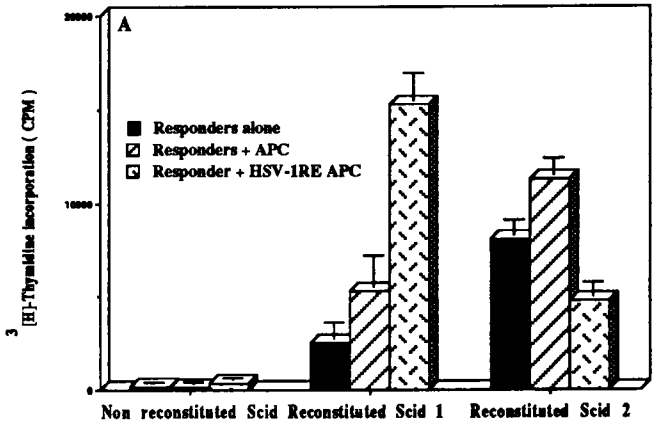
Serial two fold dilution of the purified HSV-1RE immune and naive donor cell population were stimulated with or without HSV-1RE infected syngeneic stimulator cells as described previously, and the lymphoproliferation was analysed as mentioned. Immune Donor cell population was collected from the cervical and retropharyngeal lymph nodes of the BALB/c mice that were infected a week earlier with HSV-1 RE. The naive donor cells were prepared from the spleen.

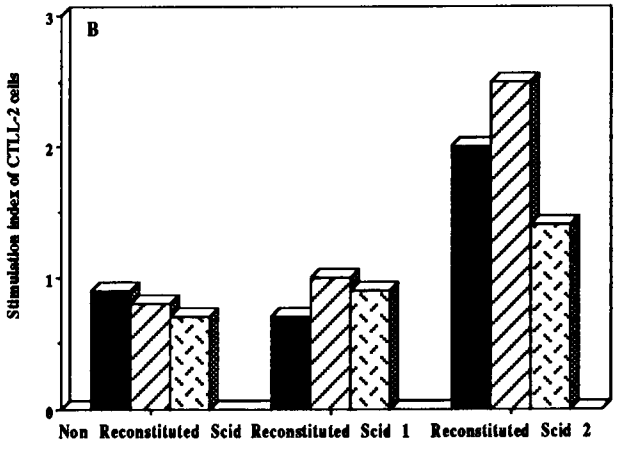
Fig.10

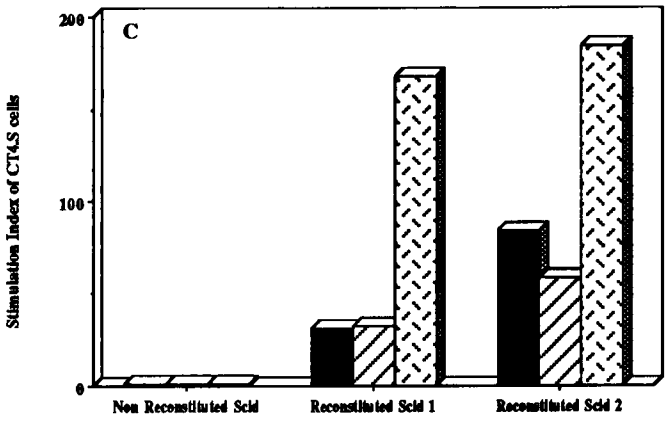
Cytokine secretion pattern of self and HSV reactive T cells in the reconstituted SCID splenic cells.

Self reactive, and HSV specific responses of the splenic cell population in the reconstituted SCID mice and secretion of IL-4 and IL-2 in the culture supernatant. Splenic cells of the SCID mice that were either non reconstituted (n=1) or reconstituted with naive (n=3) and immune CD4+ T cells (n=2) were analysed individually at the termination of the experiment. αβ TCR+ T cells in reconstituted SCID 1 (naive cell recipient) and 2 (immune cell recipient) were 73% and 78% respectively. The responder cells were prepared as described in the methods and stimulated with fixed amount of stimulator cells and various responder cell number.

A) the proliferative response of the 5 x 10^4 responders to 10^5 stimulator cells in the triplicate culture. Prior to addition of the [^{3}H]-thymidine at the end of 3 day culture, 50 µl of the culture supernatant was collected from each well in two replica plates and assayed for IL-2 and IL-4 production in 5 X 10^3 B) CTLL-2 and C) CT4.S cells, respectively. The cytokine response is presented in stimulation indices (S.I.), since two different cell types were used. S.I. was calculated as average CPM of CTLL-2 or CT4.S + culture supernatant / average CPM CTLL-2 or CT4.S + media. Approximately, 10-20 stimulation index were obtained with 1 U of rIL-2 or IL-4 in standardization experiments.


A
Responders alone
Responders + APC
Responder + HSV-1RE APC
[H]-Thymidine incorporation (CPM)
Non reconstituted Scid
Reconstituted Scid 1
Reconstituted Scid 2


B
Stimulation Index of CTLL-2 cells
Non Reconstituted Scid
Reconstituted Scid 1
Reconstituted Scid 2


C
Stimulation Index of CT4.S cells
Non Reconstituted Scid
Reconstituted Scid 1
Reconstituted Scid 2

population that either exhibited HSV specific response (Fig.10A, SCID 1) or strong self reactivity (Fig.10A, SCID 2) compared to the levels in response to syngeneic stimulator cells or auto proliferation. It is interesting to note that even IL-4 producing responders were evident in the recipient spleen, such cells were not represented in the cornea. The results may serve to indicate that different populations of cells, secreting different cytokines home in the recipient environment, which differ in the their response at both functional as well as cytokine secretion pattern.

5. DISCUSSION

HSK appears to represent an immunopathological response to viral infection. The pathogenesis of HSK in murine models involves $CD4^+$ T cells but the molecular events of the disease remain unresolved and controversial. Previous studies on euthymic animal models have implicated $CD4^+$ Th1 cells in the pathogenesis (Niemilatowski and Rouse, 1992, Hendricks, et. al., 1992, and part III) In contrast, adoptive transfer of $CD4^+$ HSV specific Th2 clones were demonstrated to induce HSK (Jayaraman et. al., 1993). However, as demonstrated in this section, adoptive transfer of HSV immune and naive $CD4^+$ T cells into SCID mice result in selective activation of $CD4^+$ Th1 cells in the pathological cornea as in euthymic mice. Furthermore, the inflammatory T cells isolated from HSK lesions were partially anergic to HSV specific proliferation without any impairment in cytokine secretion, even though HSV specific proliferative response was evident in peripheral lymphoid cells of the recipients. In addition, self reactive T cell responses were also evident in T cells collected from peripheral lymphoid tissue of both euthymic as well as in reconstituted SCID mice in response to HSV infection.

Although weak spontaneous coexpression of IL-2 and IL-4 but not IL-5 or IL-10 mRNA was evident when higher number of immune donor cells were used for reconstitution, frequently dominant expression of only IFN–γ mRNA was evident in the inflammatory cornea . This is despite the presence of both Th1 and Th2 cytokine mRNA expression in the immune donor cell population used for reconstitution. A similar responses were also evident in the naive donor cell reconstituted recipient corneas, as well. It is not known whether the dominant expression of IFN–γ mRNA seen in the immune $CD4^+$ T cell reconstituted SCID mice cornea is due to selective homing of CD4+Th1 cells differentiated in the donor BALB/c mice, since isolation of inflammatory cells from the immune $CD4^+$ T cell recipient corneas were unsuccessful due to technical reasons. However, as demonstrated in the naive $CD4^+$ T cell recipient corneas, both Th1 and Th2 cytokine mRNAs could be induced following mitogenic stimulation, although IL-10 mRNA expression was at lower levels in relation to others. Nonetheless, cytokine secretion is confined to only $CD4^+$ Th1 cells, and induction of IL-2 and IFN–γ secretion varied depending upon the corneal cell culture as well as the stimuli used. The secretion of IL-2 in response to

mitogenic stimulation in cultured corneal infiltrates may be representative of *in vitro* expanded Th1 precursors. However, secretion of IFN-γ but not IL-2 in response to HSV specific stimulation from both fresh as well as cultured corneal cells indicate antigen specific activation of primed effector cell population (Constant et. al., 1994). Many investigators have found that induction of IL-4 mRNA following *in vitro* stimulation does not result in *in vitro* cytokine secretion (Powers et. al., 1988, Betz and Fox, 1990) and some suggested that it is due to lack of maturation of IL-4 producing cells (Dekruyff et.al. 1992, Troutt and Kelso, 1993 and Groves et. al., 1994). However, presence of mature IL-4 producers were evident in recipient spleen. Thus taken collectively, a) the *in vivo* spontaneous Th1 mRNA expression as well as cytokine secretion in *in vitro* stimulation, and b) the induction of non secreted cytokine mRNAs that were otherwise not spontaneously expressed in the inflammatory cornea suggest the following conclusion. Multiple cytokine producing donor cells home to the cornea but selective activation of $CD4^+$ Th1 cells occur within the corneal milieu. Although the exact mechanisms of such selective activation of Th1 cells in the cornea is yet to be defined, one possible explanation could be that the antigen presenting cell type such as corneal Langerhans (dendritic) cells that are known to activate predominantly the Th1 response (Janeway and Bottomly, 1994, and Paul and Seder, 1994), might be playing a role. However, the role of another cytokine within the corneal milieu can not be ruled out. In this regard, expression of IL-12 mRNA, the cytokine that was demonstrated to influence the generation of Th1 effectors (Hsieh et. al., 1993 and Seder et. al., 1993) as well as turn on Th1 cytokine production in the differentiated Th2 cells (Manetti et. al., 1993 and 1994), was noticed in the inflammatory cornea (Kanangat et. al., 1995)

However, it remains unclear whether the transferred donor cells directly home in the cornea to mediate the lesions or clonally expand in peripheral lymphoid tissue of the recipients prior to their migration into the cornea. Coadministration of antigen specific T cell clones into mice at the time of infection (Alwan et. al., 1994), or into mice engineered to express the corresponding antigen (Ando et. al., 1993 and 1994) have been demonstrated to mediate immunopathological reactions in an exacerbated fashion. While T cell lines and clones represent concentrated antigen specific T cells, in the bulk polyclonal immune donor cell population the frequency of HSV specific reactive T cells are low (Prymowierz et. al., 1985) . Thus, it is not known whether the

few HSV specific reactive T cells in the immune donor cell population were amplified in recipient spleen to mediate the exacerbated response. Although in the immune $CD4^+$ T cell reconstituted SCID spleen HSV reactive T cells were seen at the termination of experiment (10-11 day post transfer), HSK lesions were manifested in much earlier. In this regard, in three immune $CD4^+$ T cell recipient spleen, donor cell population were not evident, although fulminant HSK lesions were manifested in the cornea. The results of these three mice tend to indicate that the HSK could be mediated even in the absence of T cells in the peripheral lymphoid tissue of the recipients, perhaps by direct homing in the cornea. Similarly, in the naive $CD4^+$ T cell recipients, although HSV reactive T cells were evident at the termination of the experiments (16 day post transfer), onset of HSK lesions were evident much earlier. Since in the absence of T cells HSK lesions were not manifested in SCID mice, perhaps the early onset of HSK response could be attributed to donor T cells that had gained entry into the cornea simultaneously and/or prior to their homing in the recipient spleen (to acquire HSV specific immunity to undergo clonal expansion for migration into the cornea to mediate HSK lesions). Nevertheless, it is not known whether the splenic cell population is also represented in the cornea. The T cells isolated from the inflammatory cornea although proliferated in response to mitogenic stimulation, were found to be partially anergic to HSV specific proliferative response. Furthermore, although mature IL-4 secreting cells were evident in the recipient spleen, such population was not found in the corneal cells. In addition, the self reactive cells that were noticed in the recipient spleen were also not found in the corneal cell isolate. Thus taken collectively, the cytokine profile as well as the in vitro proliferative response of the T cells isolated from the recipients, it appears that the donor cell population that home in the recipient cornea and in the spleen are different. In this regard, in absolute number, the T cells recovered from the recipient spleen represented a maximum of 25% of the input donor cell population even after clonal expansion, indicating that only a small proportion of the donor cells homed initially to the recipient spleen.

Inflamed endothelium is permissive to circulatory lymphocytes and local release of various chemotactic factors as well as local expression of adhesion molecules play a role for tissue specific migration and homing of T cells and other inflammatory cells at the local site (Hamann et. al., 1988, Nakache et. al., 1989,

Osborn, 1990, Damle and Doyle, 1990, Liversidge et. al., 1990, Mackay et. al., 1992, Mackay 1993, Bevilacque, 1993, Springer, 1994, and Oppenheimer-Marks and Lipsky, 1994). Both naive and activated lymphocytes extravasate into the inflamed endothelium and initial vascular injury is considered to be an important event in an inflammatory process. Although the migration of the T cells into the cornea is not well studied, in the initial phase of the inflammatory response predominantly activated T cells migrate into the local lesions . Thus, perhaps the initial assault of HSV infection renders the cornea : blood barrier permissive to circulatory lymphocytes for direct entry into the cornea. In this regard, the exacerbated response between different immune population could be correlated with the activation phenotype of the donor cell populations, as evident between the naive splenic and lymph node cell recipients. It remains to be seen whether adoptive transfer of $CD4^+$ T cells into asplenic recipients could result in manifestation of HSK lesions.

Coadministration of antigen during naive T cell transfer (Sunshine et. al., 1991) or following reconstitution (Sprent et. al., 1991) have been shown to result in acquired immunity in the peripheral lymphoid organs of the immunodeficient recipients. However, such immune cells were not demonstrated to be anergic for *in vitro* proliferation. Thus, the mechanism by which the corneal T cell became anergic is not known. One simple explanation is that few precursors that expand clonally and migrate into the cornea become terminally anergic at their effector site. Alternatively, the initial priming environment might influence the partial anergic state encountered. Recently several reports have described that anergy to antigen specific proliferation could be encountered if the initial activation of T cells occurs in the absence of costimulatory molecules, such as B7 or in non professional APC (Jenkins et. al., 1988 and Webb et. al., 1990). Such anergic T cells were found to be long lived and could not be activated even if necessary costimulation was provided by the professional APC. Although, the molecular details of the anergy induction is less understood (Kang et. al., 1992, Go and Miller, 1992 and Cho et. al., 1993), lack of IL-2 production for autocrine growth is considered to be a key event in induction of anergy (Kang et. al., 1992 and Janeway and Bottomly, 1994). The selective anergy to HSV specific stimulation in which IL-2 induction was not evident but induction of IL-2 and resultant proliferative response following T cell mitogenic stimulation indicate a probable block in the induction of IL-2 in HSV specific stimulation.

Antigen presentation in non professional APCs such as, endothelial cells (St. Louis et. al., 1993) and keratinocytes (Gaspari et. al., 1988) have been demonstrated to induce anergic response in the Th1 cells. The activation of HSV primed control lymph node T cells but not the HSK corneal T cells by professional APC suggest the selective state of anergy in the corneal T cells. Since inflamed endothelium is permissive to circulatory lymphocytes, it is possible that the partial anergic state of the corneal T cells might be due to priming of naive T lymphocyte within the corneal milieu. Even so, IFN–γ could be induced from the partially anergic T cells with HSV specific stimulation. Costimulatory molecules appear to play a minimal role for IFN–γ production as demonstrated by cell free antigen presenting system (Takeshita et. al., 1995) as well as in B-7 deficient APC (Bucy et. al., 1994). Similarly, secretion of IFN–γ in the *in vitro* induced anergic T lymphocytes was also observed by others (Williams and Unanue, 1991, Kang et. al., 1992 and Go and Miller, 1992). Despite partial anergy to HSV, excessive amount of IFN–γ production was observed by as little as 1.5 X 10^4 T cells recovered from the inflammatory cornea in response to HSV specific and mitogenic stimulation, suggesting maximal out put in the local environment. Similarly, high frequency of IFN–γ mRNA expression (4-6%) at the inflammatory cornea was also evident when the expression levels were compared in relation to the β-actin mRNA, as observed previously in the euthymic mice (part III).

Although $CD4^+$ Th1 cells exerted a pathological effect in the corneal milieu, protection from viral encephalitis was also evident in the $CD4^+$ T cell reconstituted SCID mice. Peripheral infection of HSV results in rapid clearance of virus from the local site and in the immunocompetent host latent infection is established in the regional ganglion within 5 days without any adverse effect (Cook et. al., 1974 and Whitley et. al., 1990). Such protection from the virus mediated neural tissue damage was demonstrated to be mediated by $CD8^+$ T cells through non cytolytic mechanisms (Nash et. al., 1987, and Simmons and Tsharke, 1992). However, as demonstrated in this section, $CD4^+$ T cell reconstituted SCID mice were also protected from viral encephalitis, unlike the non reconstituted animals. Interestingly, such protection was mediated not only by immune $CD4^+$ T lymphocytes, but also by naive $CD4^+$ T cells. Similarly, protection from CNS viral infection was also demonstrated by Wittum-Hudson and colleagues (1985) in nude mice by adoptive transfer of naive T cells ($CD4^+$ and $CD8^+$) during intraocular inoculation of HSV. Although the effect of

adoptively transferred $CD4^+$ T cells in the brain is not analyzed, it is possible that in the absence of $CD8^+$ T cells, the $CD4^+$ T cells could compensate for the virus mediated neural tissue damage. Alternatively, local production of IFN–γ from the $CD4^+$ Th1 cells that exerted the pathological effect in the cornea could effect a negative role by direct antiviral action in the local site and reduce the initial viral load that is critical for succumbing to encephalitis (Morrison and Knipe, 1994). In support to such a notion, intraocular inoculation of virulent strain of HSV-1 in normal mice along with IFN–γ or in transgenic mice expressing IFN–γ in the ocular environment abrogated viral encephalitis (Gieger et. al., 1994).

Infectious agents have been demonstrated to break peripheral T cell tolerance by activating T cells in non conventional manner (Oldstone et. al., 1991, Ohashi et. al., 1991, and Rochen et. al., 1992). In this regard, the self reactive T cell responses seen in HSV-1 RE infected reconstituted SCID mice as well as in euthymic BALB/c mice during the acute phase of HSV infection does suggest a possible role of an agonist either of viral origin or induced in host cells in response to viral infection in T cell activation process. The possibility of virus induced agonist in the cornea is very likely, since unlike LCMV or TMEV induced immunopathological conditions that are manifested in the CNS, the IP of HSK occurs only in the cornea and not in the brain even though CNS is infected with HSV following corneal inoculation. In this regard, in the euthymic mice progression of HSK lesions were evident even after clearance of infectious virus from the local site. The acute manifestation of HSK lesions in immune $CD4^+$ T cell recipients is partly reflective of viral antigen specific T cell responses, since presence of cognate antigen during adoptive transfer of antigen specific T cell clones or lines are known to result in exacerbated manifestation of immunopathological lesions (Ando et. al., 1993 and 1994, and Alwan et. al., 1994). However, it remains to be seen whether the ~ 5000 cells isolated from inflammatory cornea of naive cell recipients represent a local concentration of virus specific effector population or activated by an agonist, such as a super antigen. Furthermore, both freshly isolated and cultured cells , TNF-α secretion was not evident in the culture supernatant in response to mitogenic or HSV stimulation. Such observations tend to suggest that the major function of the $CD4^+$ cells in HSK is to exert their effector function through the release of pro inflammatory cytokine IFN–γ for activation of infiltrating inflammatory cells. It

remains to be seen whether the IP of HSK could be manifested in IFN–γ gene deficient mice or whether another cytokine such as TNF from could compensate for IFN–γ.

PART V

Role of CD4+ T cells Reactive Towards Non-HSV Derived Determinants in the Pathogenesis of HSK

1. ABSTRACT

The ability of autoreactive CD4+ T cells to induce HSK lesions was studied in syngeneic nude and SCID mouse reconstitution model. The autoreactive cell line, RB408/CD4, was derived from HSV infected mice and was found to be reactive towards I-E^d determinant of the syngeneic cells. *In vitro*, the cells were maintained in culture with or without rIL-2 in the presence of syngeneic X-irradiated stimulator cells. In response to *in vitro* stimulation with syngeneic stimulators, RB 408/CD4 secreted both IL-2 and IL-4 in culture supernatant. However, only rIL-2 but not rIL-4, induced proliferation of RB 408/CD4. The cytokine profile of RB408/CD4 was found to be of Th0 type and contained IL-2, IL-4, IL-10, and IFN–γ producers as determined by RT-PCR analysis. Adoptive transfer of RB408/CD4 into nude and SCID mice infected with HSV-1 RE on the cornea induced characteristics HSK lesions, as in euthymic, as well as naive and immune CD4+ T cell reconstitution models. Furthermore, as it was observed previously in the SCID mouse reconstitution model, HSK lesions were manifested in an exacerbated fashion. In addition, although both Th1 and Th2 cytokine mRNA expression was evident in RB408/CD4+ T cells, in the inflammatory cornea of the reconstituted nude mice, expression of only IFN–γ mRNA was evident. Furthermore, reconstituted nude and SCID mice not only manifested HSK, but also survived for a long period of time.

2. INTRODUCTION

Corneal infection with HSV-1 results in an inflammatory response of the corneal stroma and is considered to represent an immunopathological response to viral infection. Previous studies in euthymic (Niemialtowski and Rouse, 1992, Hendricks et. al., 1992, and part III) and reconstituted SCID mouse (part IV) models have implicated the role of CD4+ T cells of Th1 phenotype in the pathogenesis. However, in SCID or nude mice reconstitution models, the disease is manifested following reconstitution with naive as well as HSV immune CD4+ T cells (Russell et. al., 1984, Mercadal et. al., 1993). Such a response questioned whether the CD4+ T cell response in the cornea is mediated by non HSV reactive T cells. In support to such a notion, as mentioned in the previous section (part IV), the corneal inflammatory CD4+ T cells isolated from naive CD4+ T cell recipients fail to undergo HSV specific proliferative response *in vitro*, even though they were induced to secrete Th1 cytokines in response to HSV specific stimulation. The lack of HSV specific proliferative response indicated that either the T cells in the inflammatory cornea were non HSV reactive cells and activated by an agonist in response to HSV infection to secrete Th1 cytokines or that they were partially anergic to HSV specific proliferation.

In order to see whether non HSV reactive CD4+ T cells could induce HSK, an autoreactive T cell line with Th0 cytokine profile was used to reconstitute HSV-1 infected SCID or nude mice. As in immune or naive CD4+ T cell reconstituted mice, histologically, characteristic HSK lesions of the cornea was evident. Furthermore, when the cytokine mRNA expression was analysed in the inflammatory cornea, spontaneous expression of IFN–γ mRNA was evident as in HSV immune or naive CD4+ T cell reconstituted SCID mouse models. In addition, unlike the non reconstituted SCID or nude mice that succumbed to viral encephalitis, the autoreactive T cell line reconstituted mice remained viable for a long period of time.

3. MATERIALS AND METHODS

3. 1. Generation and maintanence of autoreactive T cell line

The autoreactive T cell line was derived from splenocytes of BALB/c mice demonstrating HSK lesions at 20 d post infection (p.i.) with HSV-1 RE on the cornea. In the initiation of culture, the splenocytes were stimulated in 6 - well plates with naive X-irradiated (3000 rads) syngeneic splenic stimulator cells with rIL-2 (5 U/ml) in LDA medium [1:1 vol/vol RPMI 1640 (GIBCO) and NCTC-109 (Sigma), 10% heat inactivated fetal calf serum, 10mM L–glutamine (GIBCO), 1mM oxaloacetic acid(Sigma), 0.2 U/ml of bovine insulin (Sigma) and 5x 10^{-5} M 2 -mercaptoethanol]. After 5 days of *in vitro* stimulation, viable cells were purified by histopaque (1083, Sigma chemicals). The presence of autoreactive T cells were observed in the expanded cell population as determined by proliferation in response to naive syngeneic stimulator cells. In the initial flow cytometry evaluation of the bulk autoreactive T cells, both CD4+ and CD8+ T cell phenotypes were evident. The CD4+ T cells were purified by CD4 subset purification column (R &D Minneapolis, MN) and restimulated for 3 passages as in initiation of culture at 5-7 day intervals. In subsequent passages, rIL-2 was omitted from the culture and the purified T cells were restimulated only with syngeneic X-irradiated stimulator cells. After 10 restimulations, growth of the cells were found to be diminshed and the cells were restimulated with or without rIL-2 after 4 days rest between passages. The cells were stable in long term culture for up to 4 months. The autoreactive CD4+ T cell line was designated as RB 408/CD4.

3. 2. Antibodies

Anti-CD4, anti-I-E, anti-IL-4 and anti-IL-2 antibodies were purchased from Pharmingen, San Diago, CA and used at 5 µg / ml. I-E reactive antibody recognizes $I\text{-}E^{d,k,p,r}$. The antibody reactive to $I\text{-}A^{d}$ (MK D6) was used as ascites fluid (1: 20 dilution). The $I\text{-}A^{k}$ reactive monoclonal antibody (Cedarlane laboratories, Ontario, Canada) was used at 7 µg /ml.

3. 3. Lymphocyte proliferation assay

Triplicate cultures of serial two fold dilutions of the purified RB 408/CD4+ T cells were stimulated with a constant amount (1- 5 X 10^4) of naive syngeneic X-irradiated splenic cells in 96 well round bottom. After 3 days in culture, the cells were pulsed with 0.5-1 μCi of [^{3}H]- thymidine over night and proliferation was measured as per the standard protocol by counting the [^{3}H]- thymidine incorporation. The IL-2 or IL-4 secretion by RB408/CD4 was measured after stimulating the cells with syngeneic stimulators for 48 hrs. The IL-2 and IL-4 were measured by growth factor dependent growth of 3000 CTLL-2 and CT4.S cells, respectively in triplicate culture. Anti-IL-4 antibody was added to the CTLL-2 cells to block the IL-4 dependent growth. Recombinant IL-2 and IL-4 dependent growth of RB 408/CD4 was measured by adding varying concentrations of recombinant cytokines to 2.5 X 10^4 cells in triplicate culture.

3. 4. Mice

Four to five weeks old BALB/c $^{nude/nude}$ (Harlan Sprague Dawley, Inc. Indianapolis, IN) and CB-17(H2^d) $^{SCID/SCID}$ (Taconic, German Town, N.Y.) were used as recipients The SCID and nude mice were housed in sterile microisolator cages. All food, water, bedding and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. All experimental procedures were conducted according to the association for research in vision and ophthalmology (ARVO) resolution on the use and care of laboratory animals.

3. 5. Corneal infection and clinical observation

The corneal surface of the deeply anesthetized mice (methoxyflurane [Metofane]; Pittman-Moore, Mondelein, Ill.) was scarified with a 27–guage needle and 5 x 10^6 50% tissue culture infective dose ($TCID_{50}$) HSV-1/ RE strain grown on vero cells was applied in 5 μl volume and gently massaged with the eye lids a day prior to reconstitution. Histopaque purified 1-5 x 10^6 RB 408/CD4 cells / mouse were transferred i/v. To increase the sample size, the recipient animals were infected on both the corneas. In some experiments, non reconstituted virus infected SCID mice were used as control. Animals were examined at different days p.i. by slit lamp biomicroscope (Keeler Instruments, Broomall, Pa.) and the severity of the stromal

keratitis in each cornea was recorded with the following scoring system as described elsewhere for euthymic mice (Newell, et al., 1989a and 1989b): (0), clear cornea; (1+), mild corneal haze; (2+), moderate corneal opacity or scaring; (3+), severe corneal opacity with visible iris; (4+), opaque cornea, iris not visible; (5+), necrotizing stromal keratitis.

3. 6. Reverse transcription and polymerase chain reaction

RNA was isolated from the autoreactive CD4+ T cells or from the reconstituted nude mouse cornea as described earlier (part IV). Eyes that showed necrotizing lesions were excluded for RNA isolation and used instead for histological evaluation. Corneas that showed clinical lesions of 0 and 3/4+ score were used for cytokine mRNA analysis by qualitative RT-PCR.

4. RESULTS

4. 1. General remarks and characterization of RB 408/CD4

In an attempt to expand the HSV reactive T cells for HSV specific cloning, splenocytes of BALB/c mice demonstrating HSK lesions were initially cultured with rIL-2 and naive syngeneic X-irradiated splenic feeder cells. In the bulk split cultures of the initially expanded population, a continuous autoreactive T cell line designated as RB 408 was encountered after three restimulations. Flow cytometry analysis of the RB 408 line indicated both CD4+ and CD8+ T cells. The CD4+ T cells were purified from the parental line, and designated as RB 408/CD4 and maintained as described in methods. As illustrated in Fig.1, the RB 408/CD4 T cell line proliferated in response to X-irradiated syngeneic stimulator cells and the response could be blocked with anti-CD4 antibody. Furthermore, as with many autoreactive T cells (Finnegan et. al., 1990), the autoreactive response was restricted by MHC encoded I-E^d allele, since only antibody reactive to I-E^d but not the I-A^d or I-A^k could block the autoreactive proliferative response (Fig.2).

4. 2. Cytokine pattern of RB 408/CD4

Although autoreactive in its characteristics, and restricted in I-E^d recognition, the RB 408/CD4 was found to contain various cytokine producers. Accordingly, when IL-2, IL-4, IL-10, and IFN–γ mRNA expression was analysed with equal amounts of cDNA samples, the presence of all the cytokines were evident (Fig.3). Murine Th1 cells produce IL-2 and use them for their autocrine growth to produce IFN–γ and/or TNF. On the other hand, the Th2 cells produce IL-4 for their autocrine growth to produce IL-10. Accordingly, the Th1 and Th2 cells use their respective growth factor for autocrine growth . However, when the IL-2 or IL-4 dependent growth of RB 408/CD4 was analysed using recombinant cytokines, only IL-2 supported the *in vitro* growth of the cells as that of the syngeneic stimulator cells and IL-4 failed to do so (Fig.4). This is despite the presence of IL-4 producing cells in the split culture used for mRNA analysis (Fig.3). However, when the split cultures were stimulated with syngeneic stimulators, induction of IL-2 and IL-4 was evident in the culture supernatant as determined by IL-2 dependent growth of the CTLL-2 cells,

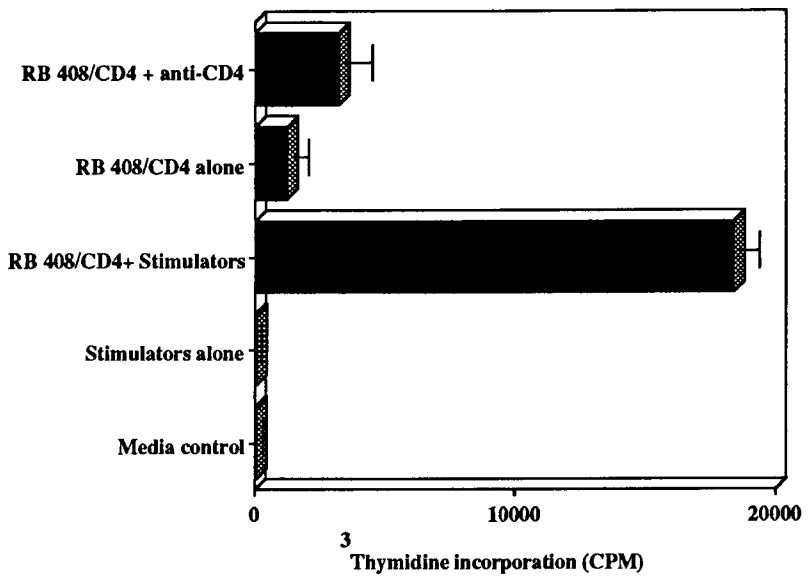


Fig.1

Autoreactive responses of RB 408/CD4

Purifed 10^4 RB 408 /CD4 cells were cultured in triplicates with or without $4X10^4$ X-irradiated syngeneic splenic stimulator cells containing either anti-CD4 antibody (5 μg/ml) or none. The proliferation of the cells was determined as described in methods.

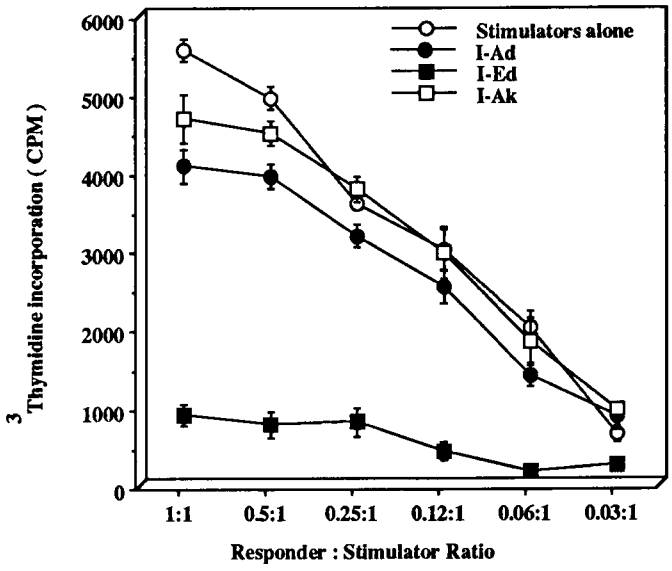


Fig. 2

IE-^{d} reactive response of RB 408/CD4

Serial two fold dilution of 5X10^{4} RB 408/CD4 was stimulated with a constant number (5X 10^{4}) X-irradiated naive syngeneic splenic stimulator cells in triplicate culture containing either none or anti-mouse I-A^{d}, or I-E$^{d, k, p, r}$ or I-A^{k} reactive antibodies as described. The proliferation of the cells were determined as mentioned in methods.

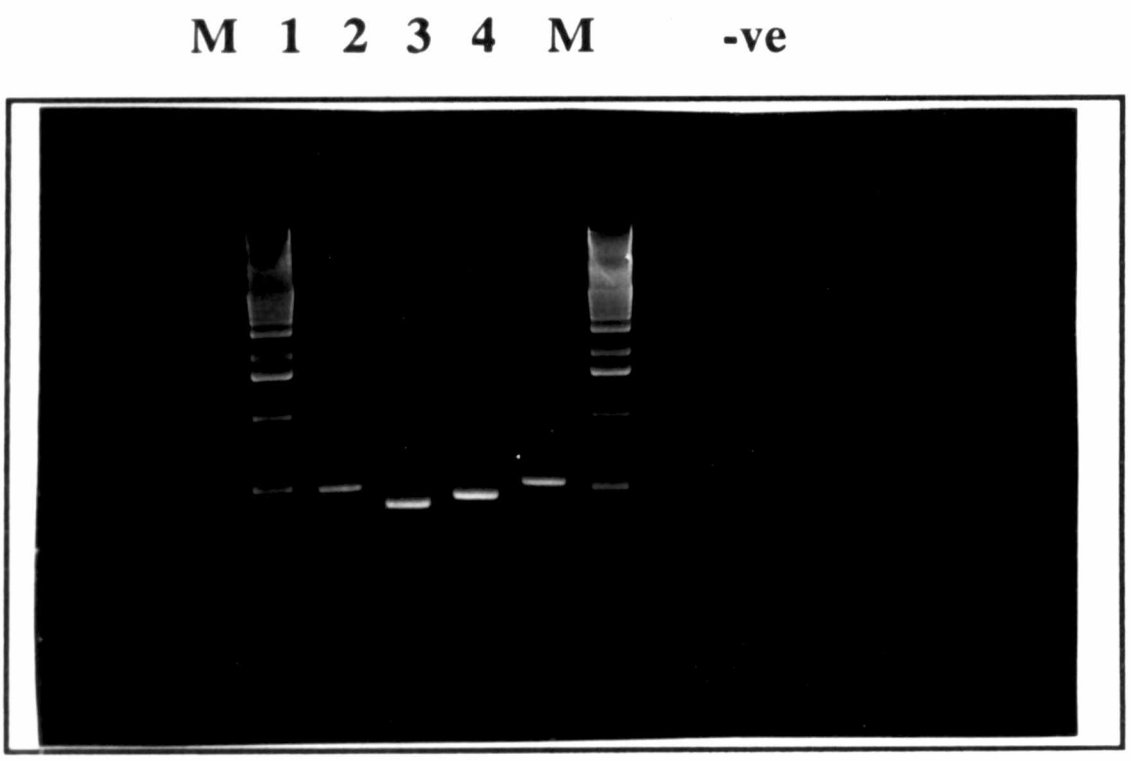


Fig. 3

Cytokine profile of RB 408/CD4

Qualitative RT-PCR analysis cytokine mRNAs in equal amount of cDNA sample prepared from total cellular RNA isolated from 5 X 10^5 RB 408/CD4 cells using cytokine specific primers. Amplification was carried out for 25 cycles. Lane 1: I Kb DNA ladder; lane 2: IL-2; lane 3: IL-4 ; lane 4: IFN-γ; lane 5: IL-10

Lane 6: I Kb DNA ladder; Lane 7-10 -ve control

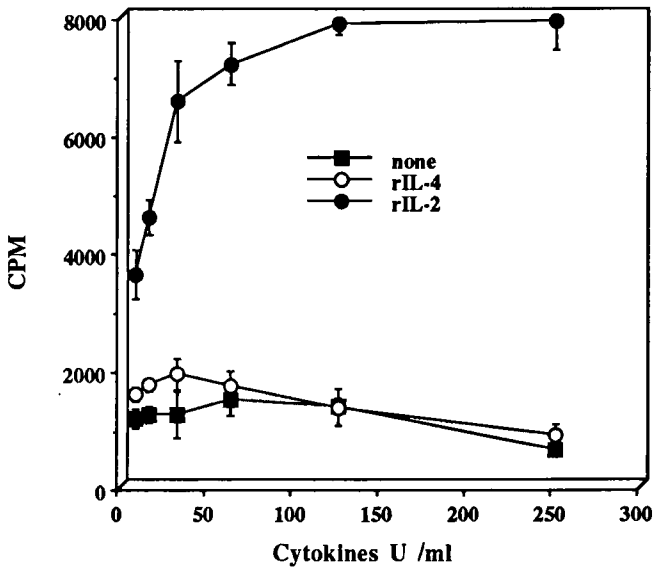


Fig. 4

In vitro growth response of RB 408/CD4 to rIL-2 and rIL-4.

2.5 X 10^4 RB 408/CD4 cells were stimulated in triplicate cultures with varying concentration of rIL-2 or rIL-4 and growth of the cells were determined as in methods.

and IL-4 dependent growth of the CT4.S cells (Fig. 5). Since CTLL-2 can grow in the presence of both IL-2 and IL-4, the detection of IL-2 in the culture supernantant was determined by addition of anti-IL-4 antibody in the culture. Furthermore, when anti-IL-2 antibody was added in the culture supernatant along with anti-IL-4 antibody, the growth of CTLL-2 cells was abrogated. These results suggest that upon stimulation of autoreactive RB 408/CD4 with naive syngeneic stimulator cells, both IL-2 and IL-4 were secreted in the culture. In addition, the response of the cells to rIL-2 but not to rIL-4 indicated that although IL-4 is produced in the culture, it is unlikely to be used as an autocrine growth factor by the RB408/CD4 probably due to lack of IL-4 receptors. On the other hand, the response of the cells to as little as one unit of IL-2 (Fig. 5) suggests the high affinity IL-2 receptor dependent growth of the cells.

4. 3. Adoptive transfer of RB 408/CD4 into HSV infected nude and SCID mice result in HSK lesions

In order to see whether the autoreactive T cell line could mediate HSK lesions, T cell deficient SCID or nude mice infected with HSV-1 RE on the cornea were reconstituted with RB 408/CD4 by i/v injection and the manifestation of HSK was monitored. As illustrated in fig. 6, reconstitution with autoreactive CD4+ T cell line induced HSK. However, early cellular infiltration was evident in the cornea following reconstitution with autoreactive T cell line. Although the early cellular infiltration was scored with the scale used for euthymic mice, the clinical appearance of the cornea was atypical, with minimal evidence of morphological damage. There was a reduction in the magnitude of initial cellular infiltration in the ensuing few days and clinical lesions typical of HSK lesions seen in euthymic mice begin to appear subsequently. However, as reported previously in the reconstituted models of HSK (Mercadal et. al., 1993, Jayaraman et. al., 1993, and part IV), exacerbated response of HSK lesions were evident by 10-12 days post transfer. In subsequent experiments (table 1), the animals were examined after reconstitution with RB 408/CD4 to monitor the course of the disease. In some corneas, the early cellular infiltration seen during 3-4 days post transfer was cleared and did not result in necrotizing lesions. However, in those corneas in which the cellular infiltration was sustained, necrotizing stromal lesions were eventually seen. Histological examination of the corneal sample at 23 days post transfer revealed, extensive tissue damage to the cornea with

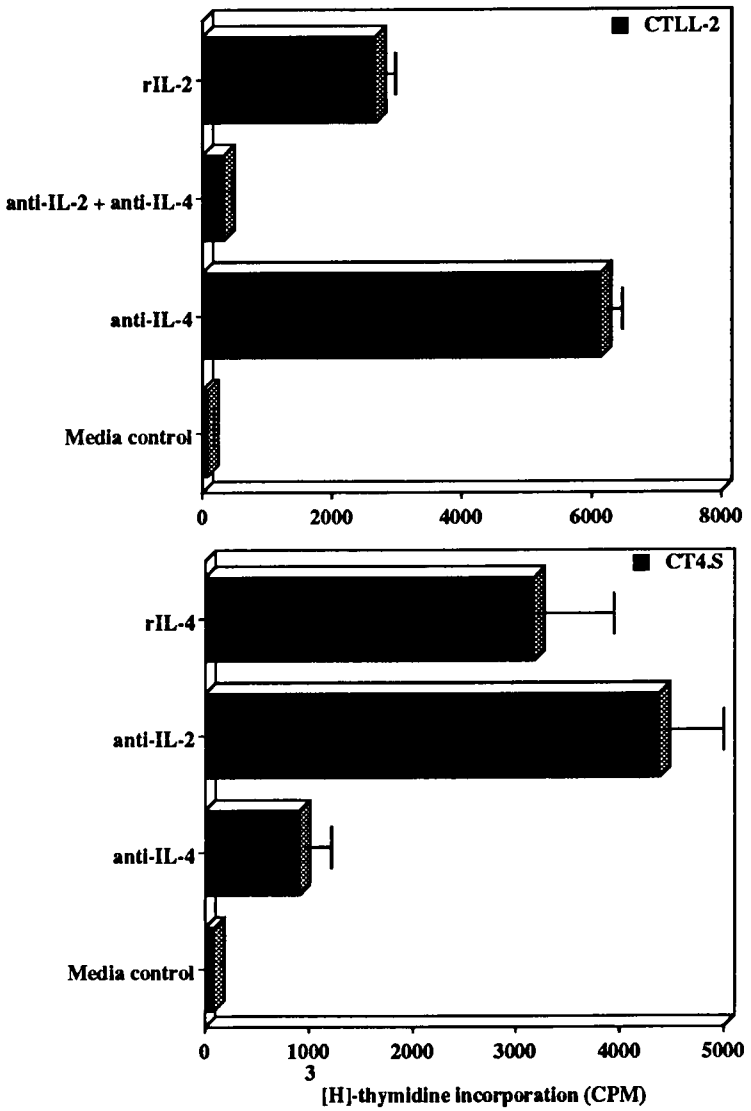


Fig.5

In vitro cytokine secretion of RB 408/CD4 in response to syngeneic stimulators.

5 X 10^4 RB408 CD4 cells were stimulated with 2.5 X 10^4 naive X-irradiated splenic syngeneic stimulator cells for 72 hrs in 24 well plates in 1 ml of medium and presence of IL-2 and IL-4 in the culture supernatant was determined by triplicate cultures in 96-well U-bottom plates containing either 3 X 10^3 CTLL-2 or CT4.S cells, respectively. The growth factor dependent growth of the respective indicator cells were determined after 72 hrs in culture. 10 U / ml of rIL-2 and rIL-4 were used as positive control. Naive X-irradiated syngeneic stimulator cell supernatant was used as media control.

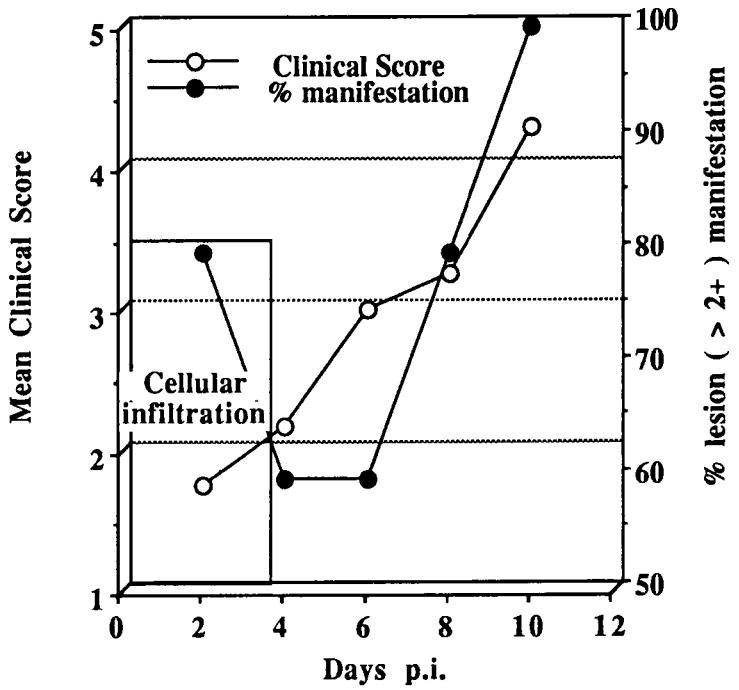


Fig. 6

HSK manifestation in RB 408/CD4 reconstituted nude mice.

HSV-1 RE infected 5 Balb/c nude mice were reconstituted with 3 X 10^6 RB 408/CD4 cells i/v, and manifestation of HSK lesions were studied. The cellular infiltration was atypical with no apparent morphological damage to the cornea and was scored in the same scale as in euthymice mice and depicted in the box. Typical HSK lesions were evident only during later time period.

Table 1

Manifestation of HSK in RB408/CD4 reconstituted Nude and SCID mice

Number of Cells[a]	Recipients	Number of corneas with HSK[b]	Day terminated[c]
none	Nude	0/10	12
3×10^6	Nude	9/10	12
1×10^6	Nude	7/12	30
4×10^6	Nude	7/10	45
3×10^6	SCID	3/6	30

[a] RB 408/CD4 cells were purified from the bulk culture by histopaque separation following 5-7 days stimulation with naive X-irradiated syngeneic stimulator cells and transferred i/v into the recipents infected with HSV-1 RE on the cornea. Part of the data is presented in Fig.6.

[b] Recipient animals were infected on both corneas to increase the population size and each eye was read individually and scored.

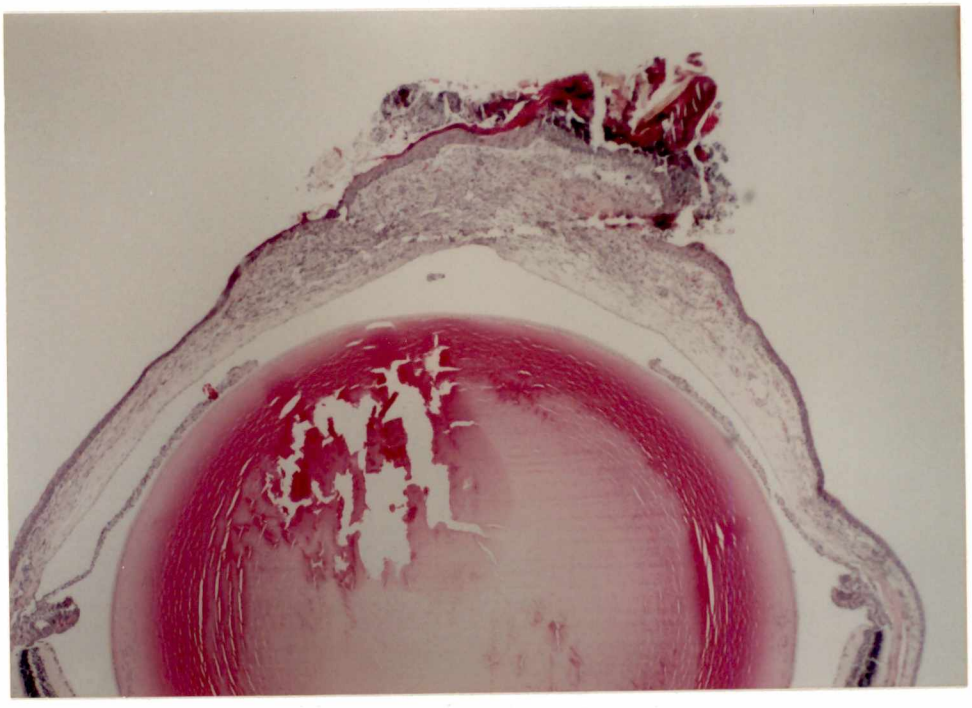
[c] One or two animals that had severe keratitis were terminated earlier and the eyes were used for histological evaluation.

vascularization (Fig. 7) as in euthymic mice as well as in HSV immune or naive CD4+ T cell reconstitution. In addition, as summarized in table 1, RB 408/CD4 reconstituted SCID and nude mice survived for a long period following corneal infection with HSV-1.

4. 4. Spontaneous cytokine mRNA expression in non inflammatory and inflammatory cornea of RB408/CD4 reconstituted mice

In order to see whether the cytokine profile of the inflammatory cornea is skewed towards Th1 or Th2 pattern, cytokine mRNA expression was analysed in two different experimental samples collected at 15 days post transfer. RNA was isolated from 3 corneas of reconstituted nude mice that had either sustained clincal lesions or did not show any clinical lesions. The results of one such experiment is illustrated in Fig. 8. Although the donor cells contained IL-4, and IL-10 producers in addition to IL-2 and IFN–γ (Fig.3), in the pathological corneal samples, only expression of IFN–γ was evident. In the clinically normal cornea neither Th1 nor Th2 cytokine mRNAs were present. In earlier studies in SCID mice with HSV immune CD4+ T cell reconstitution, the donor cell population were similarly Th0 in characteristics, although Th1 response was dominant (part IV). Nevertheless, when the cytokine mRNA expression was analysed in the reconstituted SCID mice cornea, spontaneous expression of IFN–γ mRNA was seen even though the donor cell population contained various cytokine producers. Similar response was also evident with the naive CD4+ T cell reconstituted SCID mice corneal cells both at spontaneous mRNA expression and *in vitro* cytokine stimulation, even though the cells were capable of producing various cytokine mRNAs after polyclonal stimulation. Thus it appears eventhough RB 408/CD4 T cell line is autoreactive, and contained both Th1 and Th2 producers, in the inflammatory cornea only Th1 cytokine mRNA expression was evident. Whether it is due to selective homing of IFN–γ producers or selective activation of Th1 cells in the corneal milieu is not known. However, both the posibilities can not be ruled out.

A



B

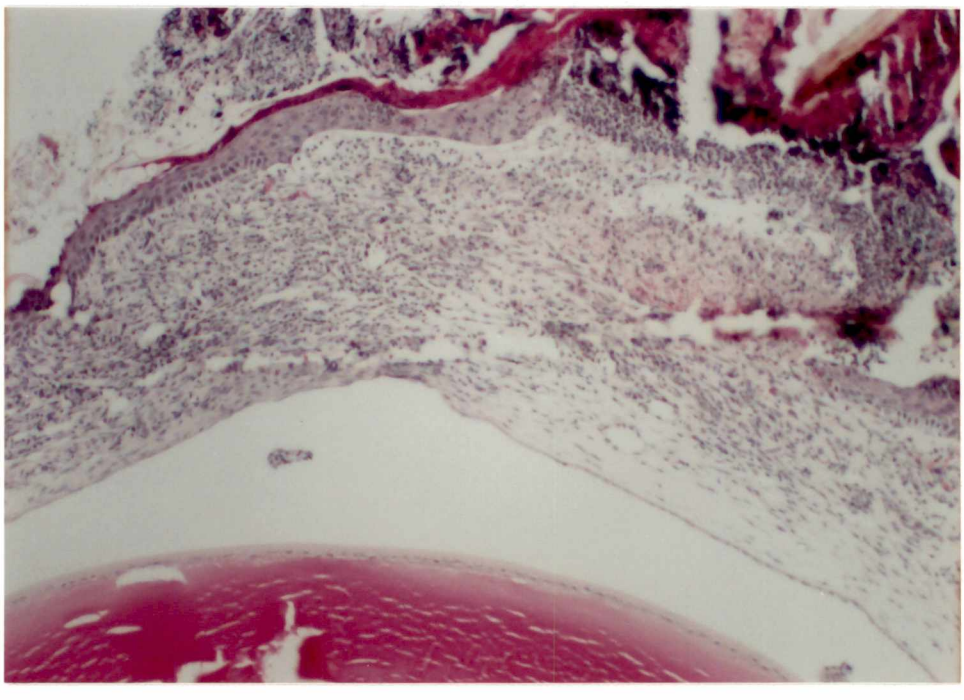


Fig. 7

Histology of HSK lesions in RB 408/CD4 reconstituted nude mice.

Eyes were collected at 23 d p.i.(22 d post transfer) following reconstitution with 408/CD4 cells.. Original magnification A) 4X B) 10 X

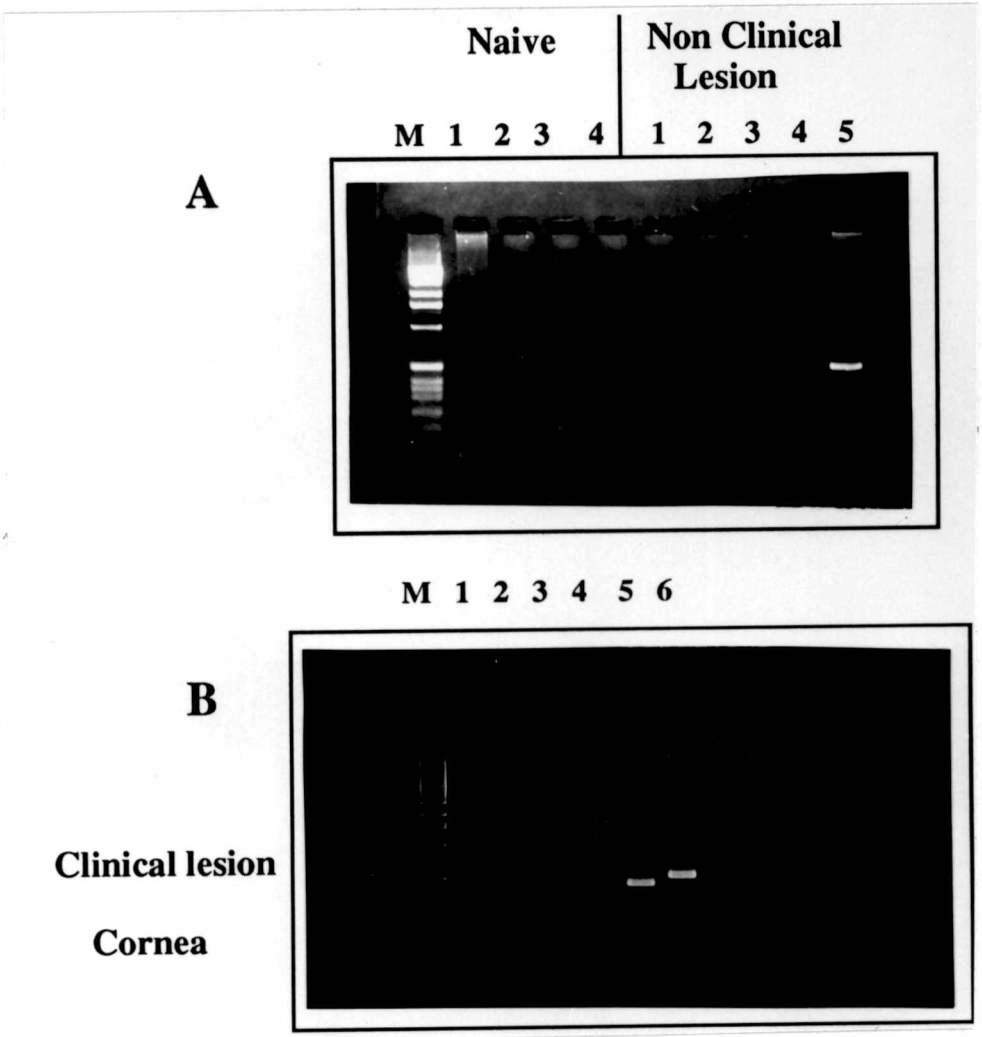


Fig. 8

Cytokine profile in RB 408/CD4 reconstituted nude mice inflammatory and non inflammatory cornes.

3 corneas were collected from nude mice manifesting HSK lesions or none at 30 day post transfer following reconstitution with 1 X 10^6 RB 408/CD4 cells. Total cellular RNA was isolated and cytokine mRNA expression was analysed by qualitative RT-PCR as described.

Panel A; Lane 1, I Kb ladder, lane 2- 5, RNA from the naive cornea, 6-9 non clinical lesion cornea amplified for IL-2, IL-4, IL-10, and IFN–γ cytokine mRNA detection; Lane 10, β-actin of the non clinical lesion cornea.

Panel B: Lane 1 I Kb DNA ladder, Lane 3-7 clinical lesion cornea amplified for IL-2, IL-4, IL-10, IFN–γ and β-actin mRNA detection. Con A stimulated T cell RNA was used as positive control.

5. DISCUSSION

Adoptive transfer of autoreactive CD4+ T cells, RB 408/CD4, into SCID or nude mice infected with HSV-1 RE on the cornea resulted in characteristics HSK lesions. Although derived from HSV primed splenic cells, as many autoreactive cells are usually encountered (Autoreactive T cells and Clones, 1990), the RB 408/CD4 line was found to be reactive towards self I-E^d determinant and proliferated *in vitro* in response to naive syngeneic X-irradiated stimulator cells. At cytokine levels, the autoreactive cell line was found to be of Th0 phenotype as determined by secretion of IL-2 and IL-4 in culture in response to syngeneic stimulators as well as by the presence of IL-2, IL-4, IL-10, and IFN–γ mRNA expression. However, *in vitro* only rIL-2 but not rIL-4 supported the growth of RB408/CD4 in the absence of syngeneic stimulators. Nevertheless, with regard to cytokine expression pattern the results of adoptive transfer experiments with autoreactive CD4+ T cell line supports the role of Th1 cytokines in the pathogenesis of HSK. (Niemialtowski and Rouse 1992, Hendricks et. al., 1992, part III and IV). Accordingly, although both Th1 and Th2 cytokine mRNA expression was evident in the autoreactive cell line used for reconstitution, in the inflammatory cornea only IFN–γ mRNA expression was evident. Although HSK lesions were manifested by CD4+ T cells, it is surprising to note that the response could be mediated by autoreactive cells. Thus, a number of mechanisms could be operational in the pathogenesis of HSK with autoreactive CD4+ T cells, none which are mutually exclusive.

Studies on the lymphocyte migration of the transferred T cell clones have indicated that the activated T cells distribute throughout several non-lymphoid tissues and showed tissue specific migration and homing (Mackay 1993). However, in contrast to the flat endothelial cells of visceral organs, normal corneal endothelium is impermissive to circulatory T lymphocytes. However, as evident by the early cellular infiltration, the initial assault of HSV infection perhaps renders them permissive to circulatory lymphocytes. Activated T cells and clones readily perculate through the walls of vascular endothelium (Picker and Butcher, 1992 and Springer, 1994) and vascular endothelial cell injury has been considered as a common inciting factor during inflammation (Bevilacqua, 1993). Normal cornea does not express MHC II and MHC I expression remains at lower levels (Wang et. al., 1987). However, in the

euthymic mice high levels of MHC I and II expression were evident as early as 1 day post infection (Cunningham et. al., 1985) and IFN–γ have been demonstrated to induce MHC II molecules in the keratocytes (Cunningham et. al., 1989). Thus, the early infiltration of poly clonal T cells containing IFN–γ could render the corneal cells MHC II positive and could serve as a sustained stimulus for activation of autoreactive RB 408/CD4 cells. In addition, corneal Langerhans cells also migrate into the central cornea during early periods of HSV infection (Miller et. al., 1993) and could function in activation of RB 408/CD4 cells resulting in subsequent inflammatory cellular infiltrates.

In euthymic mice the inflammatory response appears at the time when the infectious virus is cleared from the local site (Metcalf et. al., 1976) and CD4+ T cell infiltration into the cornea has been reported as early as 11 d p.i. in euthymic mice (Foster, et. al., 1993). However, in the SCID or nude mice reconstitution with HSV specific T cell clones (Jayaraman et. al., 1993) or immune or naive CD4+ T cells (Mercadal et. al., 1993) the inflammatory response is already in progression (exacerbated response) and the cellular infiltration was evident when infectious virus is still present. Infectious agents have been demonstrated to activate the T cells in non conventional manner and in certain viral infections super antigens have been incriminated in T cell activation (Aebischer et. al., 1990, Marrack et. al., 1991, Lafon et. al., 1992, Acha-Orbea et. al., 1993, Pantaleo et. al., 1993, and Lafon et. al., 1994). It remains to be seen whether the activation of RB 408/CD4 cells occur *via* virally encoded super antigen, and the RB408/CD4 contain a limited spectrum of T cell repertoire. Although tempting, no attempt is made in this section to link the self reactive response seen in euthymic and reconstituted SCID mice (Part IV) following viral infection to the autoreactive response of RB 408/CD4. Many investigators encountered autoreactive T cells from the antigen primed population and this regard the derivation of autoreactive RB 408/CD4 during a cell cloning was not unique. However, in subsequent studies higher number of autoreactive T cells were observed in the spleens of HSK mice and it is not known whether it is due to general breakdown in self tolerance. Further studies in this regard are needed. However, manifestation of HSK with autoreactive CD4+ T cells in response to HSK indicate that the T cell activating determinant in the cornea need not necessarily be of HSV origin.

Furthermore, reconstitution of SCID and nude mice with autoreactive T cells not only induced HSK lesions, but also protected the mice from viral encephalitis for a long period of time. Two potentially possible mechanisms could account for the protection from viral encephalitis: a) cctivated T cells readily gain entry into the brain by crossing the normal blood:brain barrier and studies on the transferred activated T cells have demonstrated the presence of T cells in the brain as early as 6 hrs post transfer (Hickey, 1991 and Hickey et. al., 1991). Thus, adoptively tansferred RB 408/CD4 line could offer protection from the viral encephalitis by migrating into the CNS due to its activated phenotype. In this regard, transfer of both naive and HSV immune T cells (CD4+ and CD8+) at the time of ocular infection with HSV has been reported to confer protection from viral encepahlitis (Wittum-Hudson et. al., 1985); b) since the virus spreads to the CNS occurs from the cornea, the early infiltration of the T cells into the cornea could reduce the viral load through anti-viral action of IFN–γ in the corneal milieu, as discussed in previously (Part IV). In addition, IFN–γ induced activation inflammatory cells such as macrophages have been demonstrated to exhibit anti-viral potential *in vitro* (Karupian et. al., 1993 and Croen, 1993) and in certain immunopathological conditions mediated by viral infection (Campbell et. al., 1994), nitric oxide has been shown to be involved in the inflammatory process. Although it is yet to be shown in murine system, bovine and human neutrophils release nitric oxide in response to IFN–γ dependent stimulation, and it is possible that the influx of neutrophil infiltration seen in HSK lesions might be playing a role not only in the inflammatory process but also in exhibiting activiral potential. In support to this notion recently, *in vivo* depletion of neutrophils in SCID mice infected in the eye has been shown to impair the clearance of virus (Tumpey et. al., 1995, and Thomas and Rouse, unpublished). Thus, the reduction in inital viral titer contributed by IFN–γ as well as inflammatory cell products during the inital phase of viral infection, although cause HSK, could perhaps account for the protection from viral encephalitis along with T cells in the CNS.

PART VI

The Role of HSV in Potentiating the Immunopathology of HSK

1. ABSTRACT

The outcome of HSK manifestation in response to topical corneal infection with inactivated and replication defective mutant viruses was studied in euthymic and reconstituted SCID mouse models. The replication defective mutants contained either a point mutation or deletion in the essential viral genes and infectious virions were grown in complementing cells. The replication defective mutants are capable of initial infection, but fail to produce infectious progeny viruses for subsequent infections. Peripheral immunization with replication defective mutants in euthymic animals induces protective cell mediated and humoral immunity as well as Th1 response similar to that of wild type virus. In contrast, topical corneal infection with U.V. inactivated or replication defective mutant viruses does not induce HSK in euthymic animals. The failure of replication defective mutant viruses to induce HSK is not due to insufficent generation of CD4+ T cell immune response, since even in the presence of higher number of wild type virus immune CD4+ T cells, infection with replication defective mutant viruses induced only a mild inflammatory lesion. Taken collectively, the data implicate that the extent of initial tissue damage in response to productive infection during the initial phase of the viral replication in the cornea, as well as the determinant density within the corneal milieu might be playing an important role in the pathogenesis of HSK.

2. INTRODUCTION

HSK, the inflammatory response of the corneal stroma, is considered to represent an IP mediated by CMI response to HSV infection. It is suspected that sporadic reactivation of HSV from the latently infected trigeminal ganglion and the resultant anterograde axonal spread to the peripheral site, such as cornea, might be the natural cause of bouts of HSK lesions seen in humans (Doymaz and Rouse, 1992). However, many investigations on murine models of HSK have been conducted on primary viral infections and have demonstrated a role for host CD4+T lymphocytes in the pathological process. Although there is some disagreement (Jayaraman et. al., 1993), as demonstrated elsewhere (Niemialtowski and Rouse, 1992 and Hendricks, et. al., 1992) and in previous sections, CD4+ Th1 cells involved in DTH reactions participate in HSK.

Although CD4+ T cells and their Th1 effector cytokine appear to play a role in the IP, initial infection of cornea with HSV appears to be a key event in the development of HSK. Accordingly, unlike certain IP of autoimmune dysfunction such as experimental allergic encephalitis (Kumar et. al., 1989) or retinal S-Ag induced uveitis (Gregerson et. al., 1990), peripheral immunization with HSV does not induce HSK lesions, but rather offer protection from keratitis during subsequent corneal challenge with the virus. Such protection is considered to be mediated by induced humoral response, rather than CMI. Accordingly, passive transfer of HSV gD specific monoclonal antibodies or hyperimmune serum at the time of primary corneal infection protected mice from developing subsequent keratitis. On the other hand, as demonstrated in adoptive transfer models, passive transfer of immune and naive CD4+ T cells at the time of primary corneal infection result in manifestation of stromal disease. However, transfer of CD4+ T cells in the absence corneal infection with HSV does not induce HSK. In this regard, HSK differs from autoimmune diseases mediated by CMI. However, while in active persistent viral IP majority of the pathological T cells are of virus specific, it is not known whether such a response is operational in HSK. In euthymic mice, following topical corneal infection, the virus is cleared from the local site before the manifestation of HSK lesions, and infectious virus is not recoverable at the site of inflammation (Metcalf et. al., 1976). Yet, viral infection is necessary to initiate HSK.

To gain insight into the role of HSV *per se* in initiating the immunopathological event, the effect of corneal infection with replication defective mutants of HSV and inactivated viruses were investigated. The replication defective mutants contained either a point mutation or deletion in the essential viral genes and infectious virions were grown in complementing cells. The replication defective mutants are capable of initial infection, but fail to produce infectious progeny viruses for subsequent infections. When the manifestations of HSK was studied in euthymic mice following topical corneal infections with either inactivated or replication defective viruses, neither of them induced an inflammatory response in the cornea. This is despite the fact that peripheral immunization with inactivated and replication defective viruses induced a Th1 response (Nguyen et. al., 1994) and are known to offer a protective CMI against HSV(Nguyen et. al., 1992 and Morrison and Knipe, 1994). However, in reconstitution models, adoptive transfer of wild type HSV immune CD4+ T cells into SCID mice infected with inactivated or replication defective viruses resulted in mild HSK lesions, while severe necrotizing lesions were observed in response to the wild type virus infection. Mild inflammatory response is seen only when higher number of cells were used for reconstitution, although such cell numbers were not necessary to mediate severe HSK lesions in wild type HSV infection. The results suggest, that CD4+ Th1 mediated immune response to HSV alone is not sufficient to initiate HSK, and perhaps extensive damage to the corneal tissue in response to productive viral replication as well as determinant density at the local site may be an important event in the pathogenesis of HSK.

3. MATERIALS AND METHODS

3. 1. Mice

Four to five weeks old euthymic BALB/c, (Harlan Sprague Dawley, Inc. Indianapolis, IN) and CB-17($H2^d$) $^{SCID/SCID}$ (Taconic, German Town, N.Y.) were used for experiments. The animals were acclimated for 5-7 days before use in specific pathogen free animal colony accredited by the American Association for Accreditation of Laboratory Animal Care. The SCID mice were housed in sterile micro isolator cages. All food, water, bedding and instruments were autoclaved or disinfected, and all manipulations were done in a laminar flow hood. All experimental procedures were conducted according to the association for research in vision and ophthalmology (ARVO) resolution on the use and care of laboratory animals.

3. 2. Viruses

The replication defective mutant virus d301 contains a deletion mutation at ICP 8 gene (Gao and Knipe, 1989) and the n504r mutant contains a nonsense mutation in ICP 27 gene (Rice and Knipe, 1990). Both the viruses are derived from the HSV KOS 1.1. strain and were grown and titrated on the complementing cells. Equal numbers of plaque forming units (pfu) of these viruses have been demonstrated to deliver approximately similar virion particles (Rice and Knipe, 1990, and Gao and Knipe, 1991) as that of wild type and was provided by Dr. David Knipe, Harvard Medical school, Boston, MA. The HSV-1 KOS derived mutant virus AN-1 was kindly provided by Dr. Bill Rochenn., School of dental medicine, Buffalo, New York, NY. The AN-1 mutant contains lacZ insertion in the alkaline exon (ICP6:lacZ) and was created by replacing the 917 bp of coding region. The mutant is defective only in packaging of viral DNA into the nucleocapsid. In Vero cells and in complementing S22 cells wild type levels of viral DNA and late viral proteins are produced. However, small amounts of infectious viruses are also produced in the Vero cells. The wild type HSV-1 KOS1.1 and RE was grown on Vero cells. Ultra violet (U.V.) inactivation of the wild type virus was carried out for 2 min. All virion preparations were prepared from cell extracts.

3. 3. Corneal infection and clinical observation

The corneal surface of the deeply anesthetized mice (methoxyflurane [Metofane]; Pittman-Moore, Mondelein, Ill.) was scarified with a 27-gauge needle and 5 x 10^5 pfu of the wild type HSV-1 KOS and RE and 1 X 10^6 pfu (prior to U.V. inactivation) of U.V. inactivated wild type viruses or 2 x 10^6 mutant viruses were applied in 4 µl volume and gently massaged with the eye lids. To increase the sample size, and to assess the function of the mutant viruses, reconstituted SCID mice were infected with wild type HSV in one cornea and with one of the mutant viruses on the other cornea. In one experiment, the HSV-1 RE (non inactivated and U.V. inactivated) was used in the euthymic mice at 5 x 10^6 $TCID_{50}$ dose. Animals were examined at different days post infection by slit lamp bio-microscope (Keeler Instruments, Broomall, Pa.) and the severity of the stromal keratitis in each cornea was recorded with the following scoring system as described elsewhere (Newell et. al., 1989a and 1989b). At the termination of the experiments eyes were collected for histological evaluation.

3. 4. Donor cell preparation and adoptive transfer

Immune donor cells were isolated from the euthymic BALB / C mice that were i/p immunized 7 days earlier with HSV-1 RE or HSV-1 KOS (5 x 10^6 pfu). Single cell suspensions were made from the splenocytes and RBCs were lysed with ammonium chloride -Tris solution (pH 7.4) and the macrophages and dendritic cells were removed by adherence on plastic surface. The T cells in the non adherent cell population were enriched by depleting the B cells by two rounds of panning on the rabbit anti-mouse IgM coated plates. The resulting T cells were further enriched for $CD4^+$ T cells using the CD4 subset column (R & D system, Minneapolis, MN) for adoptive transfer. T cell population prepared in this manner were transferred i/v to the SCID mice that had been infected with the wild type, replication defective, or U.V. inactivated viruses on the cornea one day earlier. In one experiment, control SCID mice were infected with wild type HSV-1 KOS but were not reconstituted with T cells. The enriched CD4+ T cells generally contained >80% of CD4+ T cells in various other experiments (Part IV).

4. RESULTS

4. 1. U.V. inactivated HSV fail to produce HSK in BALB/c mice

To examine the role of virus in initiating the IP of HSK, initially topical corneal infection of U.V. inactivated HSV-1RE was assessed to initiate the pathology as that of the non-inactivated viruses. Immunization with U.V. inactivated HSV has been shown to elicit Th1 response as evident by IgG2a production similar to that of the live HSV(Nguyen et. al., 1994), although at lower levels compared to live virus. Even so, the CMI response generated by U.V. inactivated viruses were comparable to that of live virus immunization (Morrison and Knipe, 1994). However, as evident in Fig.1A, topical corneal infection with U.V. inactivated virus fail to induce HSK as that of the non-inactivated virus in the euthymic BALB/c mice, and the mild lesions were confined to the corneal epithelium. However, virus specific DTH response following topical corneal infection was evident indicating the development of HSV specific DTH response (Fig.1B).

4. 2. Response of the replication defective mutants in the development of HSK in the euthymic BALB/c mice

Although HSV specific DTH response could be elicited by U.V. inactivated HSV, it is not known whether the lack of HSK manifestation with U.V. inactivated viral preparations was due to reduction in viral titer in the initial inoculum or due to viral attenuation. In this regard, the d301 and n504r mutant viruses were used to study the development of HSK response, since equal PFUs of these viruses have been demonstrated to deliver approximately similar virion particles as that of the wild type (Rice and Knipe, 1990 and Gao and Knipe, 1991). The d301 lacks the ICP8 early protein of HSV. This β gene product is a major viral DNA binding protein and is required for viral DNA replication and late gene ($\gamma 2$) expression. The n504r contains a nonsense mutation in the immediate early (α) protein ICP 27, and is required for DNA replication and $\gamma 2$ gene expression. Both the mutants express the protein products of α , β, and $\gamma 1$ genes. Immunization of mice with these replication defective viruses induces both protective CMI against the lethal challenging dose of wild type virus (Nguyen et. al., 1992) , as well as CD4+ T cell proliferative response

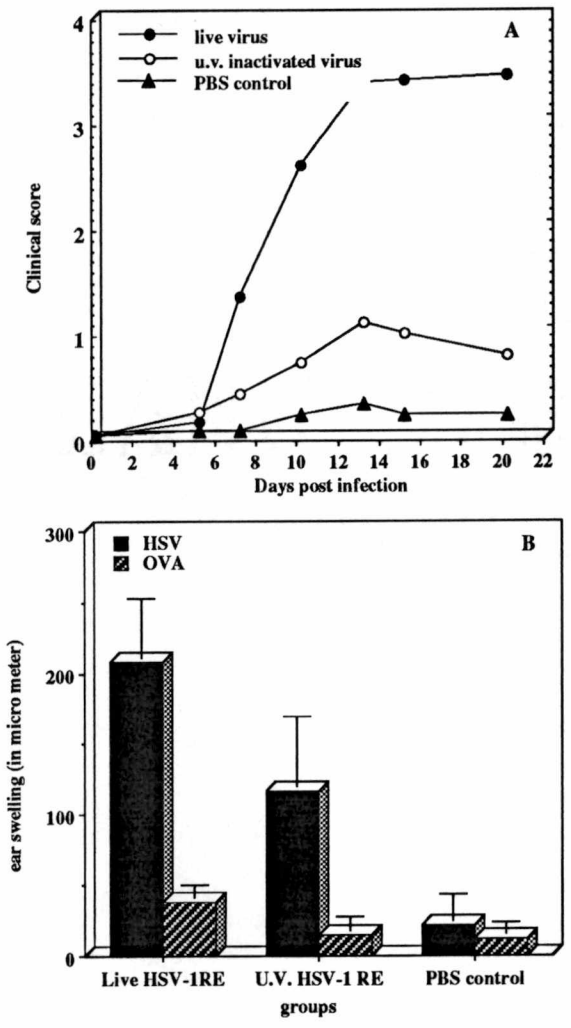


Fig.1

HSK and DTH manifestation in UV inactivated and live HSV infected BALB/c mice

A) Groups of seven euthymic BALB/c mice were infected on the cornea with the 5 X 10^6 TCID $_{50}$ Live or U.V. inactivated HSV-1 RE on the cornea. Control animals recieved PBS

B) DTH response of the Live and U.V. inactivated HSV-1RE infected mice measured prior the termination of the experiment. The mice were challenged U.V. inactivated 5 X 10^6 TCID $_{50}$ HSV-1 RE (prior to U.V.inactivation) on the ear pinnae and DTH was measured 48 hrs later. OVA was used as control antigen on the other pinnae.

(Morrison and Knipe, 1994). Furthermore, these mutant viruses have also been demonstrated to be identical in characteristics in inducing IgG sub class shift as that of the wild type, and induce a Th1 type of response (Nguyen et. al., 1994). However, topical corneal infection with the d301 and n504r mutants fail to induce HSK even when 4 times higher dose as that of the parent wild type HSV-1 KOS was used. (Fig.2) Such is the case when an another mutant virus, AN-1, was used. AN-1 produces all the gene components as that of the wild type virus including the late structural genes ($\gamma 2$), and the amount of viral DNA synthesis (replication) in normal and complementing cells is similar to that of wild type virus. However, the AN-1 is defective in packaging the newly synthesized progeny viruses and in normal infected cells empty nucleocapsids were found. The immune response to AN-1 mutant has not been studied in detail as that of other mutant viruses. However, since all viral products are synthesized, it is reasonable to expect that the mutant virus also induces the immune response similar to that of the d301 or n504r mutants.

4. 3. Response of the mutant viruses following topical corneal infection in the SCID mouse reconstituted with wild type HSV-1RE or HSV-1KOS immune CD4+ T cells

The failure of U.V. inactivated and replication defective mutant viruses to manifest HSK in euthymic mice could be attributed to lack of sufficient frequency of pathogenic T cell generation (Nguyen et. al., 1992). In this regard, in initial experiment, wild type HSV-1 RE immune CD4+ T cells were purified from the donor BALB/c mice, and 6 X 10^6 cells / mouse were transferred i/v into the SCID mice that were either infected with wild type HSV-1 RE or HSV-1 KOS on the cornea. Some animals were infected on the contra lateral cornea with the mutant (AN-1) or U.V. inactivated (HSV-1 KOS) in order to control for the reconstitution efficiency as well as to determine the response of the viruses within each mouse. As summarized in table 1, neither of the mutant viruses induced HSK lesions. In some corneas atypical clinical lesions were evident and histologically no evidence of inflammatory response was seen. Such was the case with U.V. inactivated wild type HSV-1 KOS.

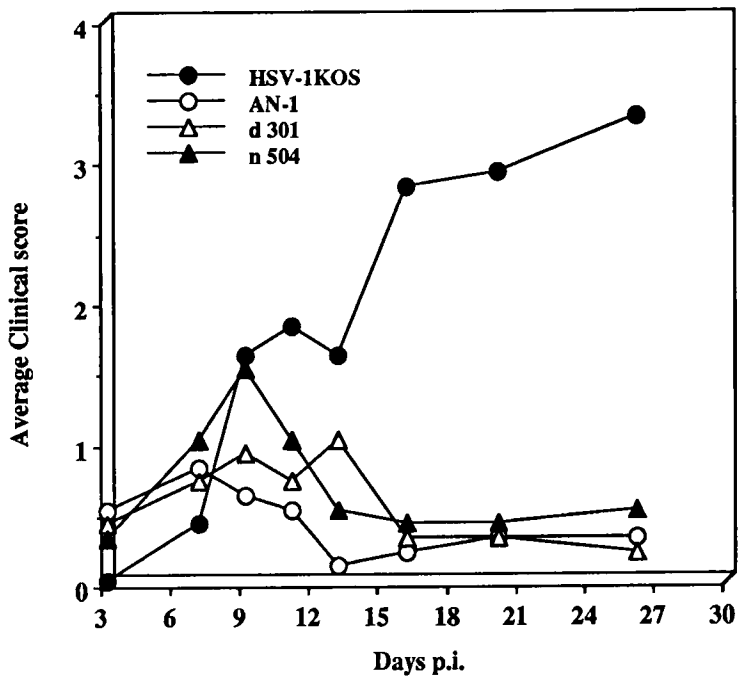


Fig. 2

HSK manifestation in BALB/c mice infected with replication defective HSV mutants

Mean clinical score of euthymic animals (n=5) infected with HSV-1 KOS1.1 and mutants.

Table 1

Summary of HSK manifestation in replication defective and UV inactivated virus infected reconstituted SCID mice

Viruses	Clinical severity	Histological appearance
HSV-1 RE(live)	5,5,5,2	MarkedPMNinfiltration with tissue damage.
HSV-1 KOS (live)	4,4,3,2	Same as above
HSV-1 KOS(U.V.)	0,0,0,1	Clear cornea
AN-1	0,0,1,1	Clear cornea
d 301	1,2,0,0*	Clear cornea, edema evident in the mild clinical lesions
n504r	2,2,0,0*	Same as above

6 x 10^6 HSV-1 RE immune CD4+ T cells from the donor BALB/c mice were transferred i/v to SCID mice that were infected with the various viruses a day prior to transfer. 4 mice per group were used. The same mice were infected with HSV-1 RE (5 X 10^5 pfu) in one cornea and AN-1 HSV-1 KOS (2x 10^6 pfu) on the other. The d301 and n504r mutants (2 X 10^6 pfu) were inoculated on both the corneas to increase the population size in two groups of mice. The results represent the summary of the clinical score of the individual cornea at the termination of the experiment on 22 d post T cell transfer. Eyes were collected individually and the clinical score lesions were evaluated histologically.

* The mild clinical lesions seen in d301 and n504r were atypical and the scoring scale was used for monitoring the response.

However, in the ipsilateral cornea that were infected with wild type HSV-1 KOS or HSV-1RE, HSK lesions were manifested. Furthermore, it is also interesting to note that in reconstituted models, corneal infection with HSV-1 KOS strain did not appear to influence the out come of HSK lesions mediated by HSV-1RE primed CD4+ T cells. In addition, in contrast to the notion infection with HSV-1 KOS strain induces a CD8+ T cell mediated HSK (Hendricks et. al., 1989a and 1989b), reconstitution with CD4+ T cells induced HSK lesions in HSV-1 KOS infected cornea. Such observation add support to the notion of mouse strain influence in T cell subset participation (Akova et. al., 1993).

Since the mutant viruses were derived from the HSV-1 KOS, it is not known whether the lack of HSK manifestations seen in the mutant viruses were due to the antigenic strain differences used to prime the immune donor CD4+ T cells, although such differences were not noticed between wild type viruses. In this regard, HSV-1 KOS primed immune CD4+ T cells from the BALB/c donors were used to reconstitute SCID mice. In addition, higher cell numbers (1.4×10^7 cells / mouse) were used so as to increase the frequency of HSV-1 KOS specific lymphocytes, although such higher number of cells are not necessary to mediate HSK in response to wild type HSV infection (table 1) (Part IV). Even so, as summarized in table 2, the mutant viruses, d301 and n504r fail to induce severe HSK lesions. In addition, as it was evident with HSV-1 RE primed CD4+ T cells, HSV-1 KOS immune CD4+ T cells also induced HSK in HSV-1 RE infected cornea. Although, severe HSK lesions were not ultimately manifested by the mutant viruses, mild clinical lesions were noticed in few cornea. Histological examination of such corneas contained mild infiltration of neutrophils confining primarily to the peripheral cornea and few T lymphocytes. However, damage to the corneal tissue was not evident (table 2).

Table 2

Summary of HSK manifestation in replication defective and UV inactivated virus infected SCIDmice reconstituted with high cell numbers

Viruses	Clinical Severity	Histological appearance
HSV-1 RE(live)	5, 4, 4	MarkedPMN infiltration, tissue damage
HSV-1 KOS(live)	5, 4, 4	Same as above
d301	2,2, 0,0,0,0	Few PMN, and T cells in mild clinical lesions
n504r	2,1,0,0,0,0	Same as above
AN-1	ND	----

1.4×10^7 HSV-1 KOS immune CD4+ T cells from the donor BALB/c mice were transferred i/v to the SCID mice that were infected with the various viruses a day prior to transfer. 3 SCID mice per group were used. The same mice were infected with HSV-1 RE in one cornea and HSV-1 KOS (5×10^5 pfu) on the other. The d301 and n504r mutants (2×10^6 pfu)were infected on both the corneas of seperate groups of mice to increase the population size. Summary of the clinical score of the individual cornea at termination of experiment on 18 d a post transfer. ND, not done.

5. DISCUSSION

Corneal infection with HSV induces a typical inflammatory response in which host CD4+ T lymphocytes appear to play a role in BALB/c animal model regardless of infection with HSV-1 RE or KOS strain of virus. (Niemialtowski and Rouse, 1991 and Akova et. al., 1993). Previous studies in the euthymic animal models implicate CD4+ Th1 cells in the pathological process (Niemialtowski and Rouse, 1992 and Hendricks et. al., 1992) and their role was further evident by both *in vivo* cytokine mRNA analysis (Part III) as well as by *in vitro* antigenic stimulation of the corneal inflammatory cells during the clinical phase of HSK (Part IV).

However, IP of HSK differs in many respect from other immunopathological conditions manifested by viral infection. In murine models of many virus induced organ specific IP, such as IP of TMEV(Peterson et. al., 1993), HBV(Mondelli et. al., 1980), Coxsackie virus (Huber et. al., 1994 and Sally and Pfaelle, 1990), or LCMV infections, inital infection with the virus at the local site appears to be a key event in initiating the pathological process, as in HSK. However, unlike these viruses that establish active persistent infection at the local site, corneal infection with HSV results in rapid clearance of virus from the local site and infectious virions are often not recoverable from the inflammatory lesions (Metcalf et. al., 1976 and Morrison and Knipe, 1994). However, the virus spreads into the CNS and establishes latent persistent infection in the neural tissues. In immunocompetent host the mild neural tissue damage contributed by initial acute neural infection (in both PNS and CNS) is demonstrated to be contained by CD8+ CTL response *via* non cytolytic mechanism (Simmons and Tscharke, 1992), although as demonstrated in the previous sections, CD4+ T cells could also mediate such protection from encephalitis. However, unlike TMEV or LCMV induced IP that is manifested at the persistent infection site where active viral replication is seen, IP of HSV infections are not manifested in the nervous system where latent persistent infection is seen and, paradoxically, CMI offers protection from viral encephalitis. On the other hand, IP is manifested in the initial site of infection, such as the cornea, even though active persistence of virus is not evident. Thus, the CMI in response to inital HSV infection appears to play a dichotomous role at the initial site of infection as well as in the distal site of persistent infection.

However, as demonstrated in this section, initial infection with virus alone at the local site in not sufficent to induce HSK. In addition to CD4+ T cells, infection with replication competent virus that produces sufficient progeny virions at the local site of infection appears to be necessary to initiate the pathological process. Although, the mutant viruses d301 and n504r are replication defective, in the initial inoculum the infectious virion particles are comparable to that of the wild type virus. Even so, the inflammatory lesions are not manifested at the local site. It is plausible that the lack of immunopathological response seen in the euthymic mice might be due to insufficient generation of the pathological Th1 response. However, in euthymic animals, the replication defective viruses (d 301 and n504r) have been demonstrated to induce both cell mediated and neutralizing antibodies characteristics of Th1 response and to offer protection from wild type viral challenge (Lien et. al., 1994 and Morrison and Knipe, 1994). Furthermore, in the wild type HSV immune CD4+ T cell reconstituted SCID mice, severe HSK lesions were not manifested in the replication defective mutant infected corneas, even when higher number of cells were used for reconstituted and higher number of infectious virion particles were used during inital infection. The lack of HSK seen in replication defective mutant viruses infected reconstituted SCID cornea is considered not due to limiting numbers of the pathological CD4+ T cells, since the donor cell population contained a sufficient frequency of T cells to induce the HSK lesions in the replication competent virus (wild type HSV-1 RE and KOS) infected corneas.

Arguably, the lack of HSK seen in the replication defective virus infected corneas might be due to a defect in late gene synthesis (Lien et. al., 1994). Accordingly, in replication defective mutants infected corneas of the reconstituted SCID mice, the non expression of late viral proteins (γ_2) might be a contributing factor in activating wild type virus immune CD4+ T cells, perhaps containing higher frequency of late gene recognizing cells, within the cornea. However, corneal infection with the AN-1 mutant virus that is defective only in the packaging of newly synthesized viral DNA, but capable of producing all the viral proteins in the initially infected cells, also failed to induce HSK in the immune CD4+ T cell reconstituted SCID mice. Similar response is also noticed in the U.V. inactivated viruses in which low titer of replication competent viruses are present.

On the other hand, the failure of mutant and U.V. inactivated viruses to induce HSK in both euthymic as well as in immune CD4+ T cell reconstituted SCID mice might be attributable to failure to generate sufficient infectious progeny viruses for subsequent productive infection of the corneal cells that were not infected during inital infection. The manifestation of HSK seen with replication competent viruses whose progeney could subsequently infect the neighboring cells favors suggest such a possibility. Although, the exact mechanisms by which the replication competent viruses induces the immunopathological reaction in the cornea are not known, the following two possibilities that cannot be mutually exclusive, could be involved.

a) The extensive tissue damage mediated by replication competent virus during inital period of viral infection might be an important factor in initiating the pathological lesions. Migration of inflammatory cells at the site of the local inflammatory response is not random and specific chemotactic factors produced at the local site were demonstrated to be involved in chemoattraction and extravasation of the inflammatory cells into the local site of inflammation (Osborne, 1990, Bevilacque, 1993 and Oppenheimer-Marks and Lipsky, 1994). For instance keratocytes , endothelial cells and fibroblasts have been shown to release various chemokines and promote chemotactic migration of monocytes, neutrophils, and facilitate the cell adhesion at the local site together with the cytokines such as IFN-γ and TNF, and IL-1. Thus, the local tissue destruction and the resultant chemokine release in response to initial viral replication during early phase might be an initiating factor in pathogenesis. Accordingly, it was shown recently that various chemotactic factors were released from the corneal stromal and epithelial cells in response to HSV infection (Su et. al., 1995).

b) Determinant density of antigens present in the local site for activation of T cells within the cornea might also be of importance. Accordingly, although peripheral immunization with replication defective d301 and n501r viruses induced protective CMI as that of the wild type virus (Morrison and Knipe, 1994), the magnitude of the cellular immunity was demonstrated to be low compared to the wild type virus (Nguen et. al., 1992). It was suggested that differences in magnitude could be accounted for viral late gene products that are not synthesized by the mutant viruses However, as demonstrated the AN-1 mutant that synthesize the late gene products but fail to package the infectious progeny virus for subsequent productive infection also

failed to induce HSK lesions even in the presence of wild type immune CD4+ T cells. Such was the case with the U.V inactivated virus. Thus, the cumulative determinant density in the corneal environment to activate the few infiltating T cells might also be an important factor for progression of the disease. However, it is not known whether such determinant density is contributed entirely of HSV origin. Release of sequestered antigen in response to infection (Sercarz et. al., 1993) as well as determinant spreading (Elson, et. al., 1995) was considered to be involved in IP of infectious diseases in immune privilegesites. The manifestation of IP of HSV infection seen in the cornea but not in the CNS does suggest that the determinants present in the local site might play an important role in the IP of HSK. Possible determinants include exposure of host heat shock protein and other host cell determinants released from corneal cells in response to cytopathic effect of HSV during initial infection as well as from the apoptotic neutrophils in the cornea.

PART VII

Conclusions and Future Perspectives

The experiments conducted in this dissertation were aimed to address the multistep immunopathological events of HSK occurring in the immune privileged cornea and to resolve the controversy, whether the disease is manifested by Th1 or Th2 cells. The following major conclusions were drawn from the results of the current study.

a) Quantification of spontaneous *in vivo* cytokine mRNA expression during the course of HSK in euthymic mice at the effector site has confirmed the observations of others that the disease is primarily mediated by CD4+ Th1 cells. Accordingly, the Th1 cytokines are involved not only in the clinical phase of HSK, but are also involved from early on in the pathogenesis. Similarly, at the cytokine level, reconstitution models of HSK appear mechanistically similar to that of euthymic animals. In addition, the corneal environment appear to influence selective activation of CD4+ Th1 cells. On the other hand, in contrast to the notion of CD4+Th2 cell involvement in the pathogenesis of HSK, the Th2 cytokines, particularly IL-10, appear to be involved in the resolution of mild clinical lesions seen in a few euthymic animals. In addition, the cytokine mRNA expression as well as secretion occurs in the corneal inflammatory cells at higher levels, and the population of cells that are represented in the peripheral lymphoid organ and in the cornea at any one phase of HSK in both euthymic as well as in reconstituted SCID mouse models are either different or behave differently.

b) The induction of HSK is not only influenced by antigen specific CD4+ T cells, but was also evident with CD4+ T cells reacting towards self MHC determinant.

c) In addition to CD4+ cells, productive progeny virion replication in the cornea appears to be necessary for inducing HSK. Infection with replication defective viruses that are incapable of making infectious progeny virions do not induce HSK, even in the presence of sufficient frequency of pathological CD4+ T cells.

At one end of the spectrum HSK represents a classical virus induced IP mediated by host CD4+ T cells activated in response to initial viral infection on the cornea. On the other end, the resultant CMI culminates in tissue damaging inflammatory response dominated by inflammatory cells. Yet, in the absence of CD4+ T cells and initial infection with replicating virus, HSK lesions were not

manifested. As with many animal models of human diseases, murine models of HSK have certain limitations. The most restricting element is the fact that pathology is initiated by acute viral infection on the cornea, while in humans the disease is considered to be mediated by reactivation of virus from latency. Nevertheless, the course of the disease in animal models has helped to clarify certain fundamental issues. However, several issues remain unresolved. For instance, a) what is the role of CD4+ T cells *per se* in the pathogenesis of HSK and b) whether their effector cytokines *really play a role* in the pathogenesis or c) the secretion of cytokine is only a collateral representation of distinct population of CD4+ cells happen to secrete Th1 cytokinesonce they gain entry into the cornea.

Differentiated Th1 and Th2 cells upon mitogenic stimulation secrete faithfully the irrespective cytokines. However, differentiation from the (undifferentiated) precursors in response to antigenic stimuli and secretion of their characteristics cytokines vary depending upon the APC type, co-stimulatory molecules, antigen system, peptide ligand, as well as the genetic make up of the host. In addition , it is of great concern whether each cell produces distinct cytokines after differentiation, or one cell produces more than one cytokine. While it is possible in *in vitro* samples to evaluate the concept with differentiated cells (clones), the movement of cells *in vivo* are dynamic, and secretion of effector cytokines at the local site is influenced by several factors as mentioned above. CMI against viruses and bacteria in both HSK susceptible BALB/c ($H2\text{-}^{d}$) and resistant C57/BL-6 ($H2\text{-}^{b}$) mice is dominated by Th1 cytokines; yet, HSK is not manifested in C57/BL-6 mice. Such a paradox indicates that the nature of immune response *alone* is not sufficient to induce HSK and demonstrates the role of host genetic make-up to inducing IP. In HSK, the genetic make-up of the host does not appear to be influenced by MHC loci, but is rather involved through non MHC associated genes. Direct evidence in this regard comes from HSK studies on the congenic BALB/c mouse strains. C.AL-20 ($IgH\text{-}1^{d}$, $H2^{d}$) and C.B-17 ($IgH\text{-}1^{b}$, $H2^{d}$) mice which differ in at least one gene locus closely linked to the constant region of immunoglobulin heavy chain, but are homologous in MHC loci. These two mice differ drastically in their ability to manifest HSK. Accordingly, C.AL 20 mice is susceptible to HSK, but C.B-17 mice are resistant. In the resistant mice, transfer of Th2 clones derived from C.AL.20 did induce HSK (mild clinical lesions, see part III). However, the exacerbated lesions seen in the

susceptible C.AL-20 and BALB/c (IgH-1^a, $H2^d$) recipient was not evident in C.B-17 recipients. In addition, C.B-17 recipients resolved the mild clinical lesions (Jayaraman et. al., 1993). Although the authors concluded that the response is mediated by Th2 cells, in the pathogenesis of HSK definitely IL-4 cytokine is not involved in the induction of IP because *in vivo* neutralization of IL-4 in euthymic mouse model throughout the course of HSK did not abrogate the pathology (Hendricks et. al., 1992). However, the disease promoting ability was found to be contained within the CD4+ T cell itself. Accordingly, transfer of C.B-17 HSV immune polyclonal CD4+ T cells into BALB/c nude mice did not promote HSK, unlike the normal BALB/c or C.AL-20 CD4+ T cells (Akova, et. al., 1993). This experiment suggests the influence of the donor cell population in disease promotion. However, it is not known whether the differential disease promoting ability seen between the polyclonal donor cells derived from the congenic mouse strains is due lack of pathogenic CD4+ Th1 cells or due to the presence of Th2 cytokine producing cells that suppress the pathogenic CD4+ Th1 cells. Taken collectively the lack of HSK pathogenesis in nude mice receiving HSV immune CD4+ T cells from resistant CB-17 mice, (Akova et. al., 1993), and failure of C57/BL-6 mice to manifest IP of HSK suggest the influence of donor cell population gene cluster in the disease promoting ability. In this connection it is questionable whether a) the Th1 response seen in HSK susceptible mice is a reflection of the natural Th1 response of the T cells entering into the cornea, or b) the pathology is mediated by the physical presence of CD4+ T cells in the cornea that upon local environment influence (IL-12) happen to produce Th1 cytokines or c) the Th1 cytokines produced by CD4+ T cells result in manifestation of HSK. For instance, it was found that anti-IFN–γ treatment during the priming phase abrogatethe development of HSK (Hendricks, et. al., 1992) but once the immune response is mobilized neither anti- IL-2 nor anti-IFN–γ treatments were effective. It is very likely that much of the effect of Th1 cytokines, (IFN–γ), might be towards induction of other gene products either from the corneal cells or from within the T cells. Similarly, Ando and colleagues (1993, and 1994) observed that hepatic tissue injury mediated by Th1 cytokine secreting CD8+ CTL could only be blocked transiently by anti-cytokine therapy, and *in vivo* neutralization of Th1 cytokines were ineffective after a narrow window of time. With regard to HSK, of particular interest are the chemokines for inflammatory cells, since in the absence of T cells sustained

migration of inflammatory cells into the cornea is not evident. Some evidence indicates that IFN–γ itself will recruit inflammatory cells, if present in the ocular environment when expressed as a transgene or whenexperimentally inoculated (Gieger et. al., 1993). However, other cytokine influences are not known and it will be of particular interest to see what will be the effect of other cytokines in IFN–γ gene deficient mice. Thus, it is clearly important to unravel the role of CD4+ T cells and IFN–γ in HSK pathogenesis in BALB/c animal model to understand whether the disease promoting ability resides within the CD4+ T cells or in its effector cytokines. Similarly, it will be of interest to analyze the cytokine secretion pattern between the C.AL-20 and C.B-17 mice following corneal infection with HSV to gain insight into the role of Th1 and Th2 cytokines and to address the role of the CD4+ T cells *per se* in the pathogenesis of HSK. Judging from the C57/BL-6 mice responses to various intra cellular pathogens, and the response of the CB-17-SCID mice to promote Th1 response in the BALB/c mouse CD4+ T cells (part IV) tend to indicate that the congenic mouse strains would also make similar cytokine profiles. It is likely that the differential response seen between the congenic CD4+ T cells might be due to deficiency in adhesion / activation molecules on T cells for homing into the cornea. Subtractive cDNA hybridization or differential mRNA amplification between C.AL-20 and C.B-17 CD4+ T cells, ideally of the same cytokine profile, perhaps would answer the question.

Similarly, the Th2 cytokine role should be defined for its potential therapeutic application. Although during the afferent phase of HSK Th2 cytokine, IL-4, was found not to be involved in the pathogenesis (Part III, Hendricks et. al., 1992), its role in the resolving phase of HSK had not been studied. In resolving phase of HSK, both IL-4 and IL-10 was found to be expressed in the corneal lesions and the role of IL-10 in the afferent phase was reported. Future studies should address whether IL-10 and IL-4 administration after the disease progression would cause the lesion to diminish or resolve. An alternate strategy to address the role of Th2 cytokine will be to study the effect of neutralization of these cytokines in those animals that manifest mild clinical lesions even after 15-20 days of p.i. Thus, it should be seen whether in these animals neutralization of either IL-4 or IL-10 or both by antibody treatment will cause severe lesion manifestation. Alternatively, IL-12 treatment in this category of animals should also be studied. In Th2 mediated schistosoma granulomatosis, IL-12

administration has abrogated eosinophil dependent IP by down regulating Th2 cytokines and increasing Th1 cytokine production. Thus, either parenteral or intra-stromal injection of IL-12 in milder clinical lesion animals should be carried out to see whether severe keratitis results after treatment. These studies will add further strength in our understanding about the role of Th1 and Th2 cytokines in the outcome of HSK.

In addition, it should be evaluated whether in T cell deficient SCID mice intra stromal injection of various T cell derived chemokines such as IP-10, neutophil activating factor (NAF) with or without T cell cytokines will recruit inflammatory cells to induce HSK lesions in the absence of CD4+ T cells and viral infection. Such a model would answer the question whether physical presence of CD4+ T cells is necessary or its effector and chemokine molecule themselves are sufficient to cause the inflammatory reactions. This model would also allow the assessment of anti-inflammatory agents such as prostaglandins or chemokine antagonist.

Another unresolved issue in HSK pathogenesis is the continued activating stimuli for the CD4+ T cells in the cornea. Although initial assault with replicating virus is necessary to initiate the IP, in euthymic mice by the time immune response is generated and T cells mobilize into the cornea, virus is cleared from the local site at least as a replicating antigen. Although immunohistochemical studies in the euthymic mice have demonstrated CD4+ T cells during early periods (5-7 d p.i.), large number of CD4+ T cells have been detected only after 11-15 days p.i.(Tamesis et. al., 1994) ,While it is possible that some of the CD4+ T cells are HSV specific and react to the persisting viral antigenic peptides, the possibilities are real that another non HSV antigen induced in response to HSV infection in the cornea might also be involved in chronic progression of the disease.

One possible antigenic candidate is the host heat shock protein (hsp)that is induced by inital HSV infection. hsp60 has been shown recently to act as an additional T cell activating determinant in inflammatory bowel disease, IDDM, SLE, and reactive arthritis and other families of hsp expression has been shown in response to HSV and Vaccinia virus infection *in vitro*. Although immunological parameters of the hsp in response to HSV infection are not known, judging from the role of this class of protein in various inflammatory response, it is very likely that hsp might be functioning as co-antigenic determinant. It should be evaluated whether in response to

HSV infection, a) hsp(s) are expressed in the cornea, and b) hsp reactive cells are noticed in HSK animals and c) whether they are expressed immediately or after progression of the disease. Similarly, the antigen presentation function need to be evaluated by stimulating HSV and CFA/hsp peptide primed T cells in reciprocal manner with HSV infected and hsp peptide pulsed stimulator cells to see whether HSV infection induces hsp(s) antigen presentation. These results will add strength to the notion of hsp as an induced determinant in HSK.

Ideally, the corneal T cells should be assessed for *in vitro* proliferative response. However, isolation of cells from cornea is often difficult. To circumvent the problem, hsp reactive CD4+ T cell clones should be transferred into HSV infected SCID mice to see whether HSK could be manifested. Since even self determinant reactive RB 408/CD4 +T cells could manifest HSK, if hsp is expressed in the cornea in response to viral infection, and act as an antigenic determinant, it might potentially activate the adoptively transferred hsp reactive CD4+ T cells in the cornea to induce HSK. In addition, it will be of interest to see if replication defective mutant viruses also induce HSK in response to hsp reactive T cells to evaluate whether determinant density or quantitative determinant expression contributed by both viral and induced determinant is important in the corneal milieu.

Finally, as mentioned throughout different sections, self reactive cells were evident in response to HSV infection in both euthymic as well as reconstituted SCID mice. Such breakdown of self tolerance has not been reported for HSV infection. It should be determined whether such self reactive T cells represent a distinct population of T cell repertoire activated by super antigen or it is a general breakdown of self tolerance.

In conclusion, the current study provide mounting evidence that in euthymic BALB/c and reconstituted mouse models that the pathogenesis of HSK involves the essential participation of CD4+ T cells with a type 1 cytokine profile. Whether the pathogenesis involves the participation of specific cell types or by their effector molecules is to be defined, due to the reasons discussed above. Similarly, the trigger for the apparent recruitment and activation of IL-10 producers remains to be identified. It is unlikely to be virus itself since such has disappeared days or weeks before regression occurs. One possible cofactor in the recovery process is TGF-β, a cytokine endogenously produced by cells surrounding the anterior chamber and

considered as involved at least in part in anterior chamber immune deviation (ACAID) induction. TGF-β has been proposed to act as a Th1 cytokine down regulator and is expressed in the normal cornea. However, whether its expression is modulated during HSK is not known, and it is possible that this cytokine might be playing a role within the corneal environment. Thus comparative analysis of IL-12, IFN–γ, TGF-β and IL-10, and perhaps IL-4 during different phases of HSK progression would shed insight into the role of different cytokines in the pathogenesis. In addition, in those animals undergoing recovery the regulatory CD8+ T cells generated in spleen, induced by the emigrated TGF-β charged APC from the ocular environment, might play a role. In this regard anterior chamber inoculation of HSV (Hendricks, et. al., 1989) had been demonstrated to ablate the development of HSK. It remains to be seen whether HSK lesions are manifested with higher incidence and severity without resolution in splenectomized euthymic mice. Similarly, it will also be of interest to see whether in asplenic T cell deficient SCID mice, adoptive transfer of immune and naive CD4+ T cells would result in HSK. Such experiments would help to comprehend whether the donor cell population home directly in the cornea or enter the cornea after clonal expansion in the spleen to mediate HSK.

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VITA

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