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Analysis of the Relationship between Viral Infection and Autoimmune Disease

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Summary

The clinical association between viral infection and onset or exacerbation of autoimmune disorders remains poorly understood. Here, we examine the relative roles of molecular mimicry and nonspecific inflammatory stimuli in progression from infection to autoimmune disease. Murine herpes virus 1 (HSV-1 KOS) infection triggers T cell-dependent autoimmune reactions to corneal tissue. We generated an HSV-1 KOS point mutant containing a single amino acid exchange within the putative mimicry epitope as well as mice expressing a TCR transgene specific for the self-peptide mimic to allow dissection of two pathogenic mechanisms in disease induction. These experiments indicate that viral mimicry is essential for disease induction after low-level viral infection of animals containing limited numbers of autoreactive T cells, while innate immune mechanisms become sufficient to provoke disease in animals containing relatively high numbers of autoreactive T cells.

Introduction

Two general explanations have been put forward to explain the clinical association between microbial infection and induction or exacerbation of autoimmune disease. One mechanism depends on evidence that the immune response to pathogens provides a nonspecific stimulus of the innate immune system that promotes activation and expansion of autoreactive T cells (Horwitz and Sarvetnick, 1999). A second holds that the pathogen itself may provide a counterfeit antigenic stimulus that provokes autoreactive T cells (Oldstone, 1987; von Herrath and Oldstone, 1996). The contribution of nonspecific inflammatory mechanisms has received extensive experimental support in several different animal models of autoimmune disease (Horwitz and Sarvetnick, 1999). The role of antigenic mimicry has also received extensive support. For example, viral peptides can cross-stimulate autoreactive human T cells, and mice that express a viral protein in a relevant target tissue develop autoimmune disease after viral infection (Hemmer et al., 1997; Wucherpfennig and Strominger, 1995; Ohashi et al., 1991). Cross-reactive T cell activation (mimicry) has also been implicated in the pathogenesis of Lyme disease (Gross et al., 1998) and a murine model of heart disease (Bachmaier et al., 1999).

The issue of self-mimicry by microbial agents has been studied in murine Herpes Stromal Keratitis (HSK), a T cell-dependent autoimmune response that destroys corneal tissue after HSV-1 KOS infection (Avery et al., 1995; Streilein et al., 1997). Viral mimicry may provoke this disorder because Th1 cell clones that initiate HSK respond to both a corneal self-antigen and a peptide derived from the UL6 protein of HSV-1 KOS. Moreover, a replication-defective HSV-1 KOS virus that does not express the UL6 protein fails to induce HSK in adoptive hosts given virus-immune T cells (Zhao et al., 1998). However, the inability of this replication-defective mutant to induce disease compared with a glycoprotein B-deficient strain may have reflected a difference in virulence between the two deletion mutants and/or inhibitory effects of low levels of UL6 expressed at the protein level by the replication-defective virus.

A direct test of the contribution of an HSV-1 peptide mimic to the development of disease after viral infection depends on generation of a replication-competent virus containing a single amino acid exchange that alters the putative mimicry epitope and analysis of mice that express a TCR transgene (C1-6) specific for a potential viral mimic (Zhao et al., 1998). We find that this exchange virtually abrogates disease induction, while disease susceptibility is increased dramatically in mice expressing the C1-6 TCR. This mutant virus and TCR transgenic mouse model are used to delineate the relative contribution of antigen-specific and innate immune mechanisms to the pathogenesis of autoimmune disease after viral infection.

Results

Generation of a Replication-Competent HSV-1 KOS Mutant Containing a Single Amino Acid Exchange

A previous comparison of several replication-defective HSV-1 mutants suggested that expression of the UL6 protein was important for induction of HSK following HSV-1 infection (Zhao et al., 1998). However, impaired replication or other functional defects of the HSV-1 KOS/UL6^{m}-mutant virus might have contributed to its failure to induce disease. A decisive test of the putative UL6-derived peptide mimic requires generation of a UL6 amino acid exchange mutant that alters the UL6 T cell epitope but spares HSV-1 replication and function.

A mutation that converts the *UL6* Ser at position 309 to Leu (S309L) and disrupts the predicted class II binding frames of the postulated mimic was induced by an alteration of AGC (encoding Ser) to CTT (encoding Leu) after site-directed mutagenesis (Figure 1A). The HindIII restriction site created by this mutation was used to moni-

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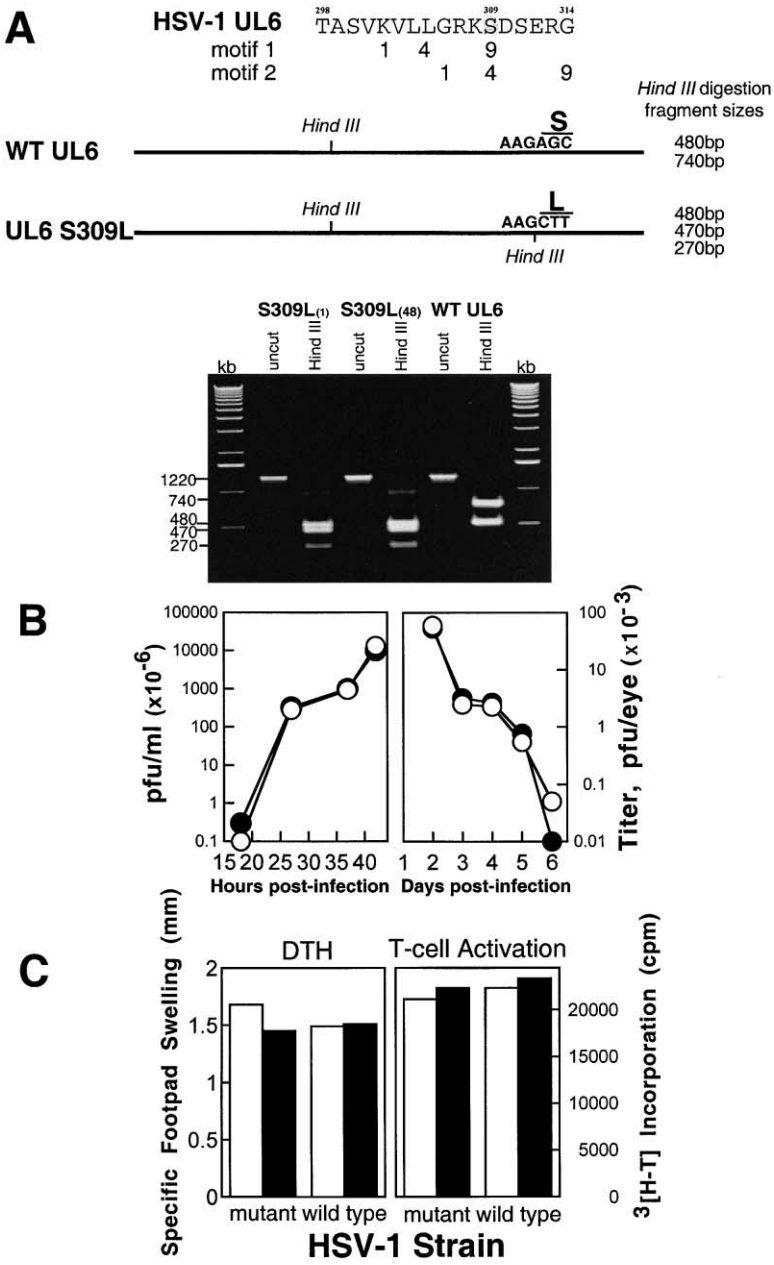


Figure 1. Generation and Analysis of KOS/UL6^{S309L}

(A) Two potential binding motifs for the UL6 peptide (aa 298–314; TASVKVLLGRKSDS ERG), based on preferred amino acid anchors and the I-A^d crystal structure (top). The introduction of an AGC→CTT mutation in an epitope-coding region of the *UL6* gene in HSV-1 KOS creates a HindIII site at bp 922 (middle). Viral DNA extracted from Vero cells infected with isolates of wild-type KOS or the mutant KOS/UL6^{S309L} (1,48) was used as template for PCR amplification of a 1.2 kb fragment of *UL6* (bottom), which was gel purified and digested to test for the presence of the HindIII site within the epitope coding region (480 and 740 bp for wild-type and 480, 470, and 270 bp for KOS/UL6^{S309L}).

(B) KOS/UL6^{S309L} replicates at the same rate as wild-type KOS. Vero cell monolayers were infected with ten viral particles of KOS (closed circles) or KOS/UL6^{S309L} (open circles) per cell and harvested at 18, 27, 37, and 42 hr postinfection (left). Samples were analyzed for virus yields as described (Sandstrom et al., 1986). Data points represent the mean of duplicate samples. Viral titers in eyes of infected mice during the period of acute replication (right). C.AL-20 mice were ocularly infected with 4 × 10^6 PFU wild-type KOS (closed circles) or KOS/UL6^{S309L} (open circles); eyes were harvested on days 2, 3, 4, 5, and 6 and assayed for viral particles as described (Sandstrom et al., 1986). Data points represent the mean of duplicate samples.

(C) The T cell response to the KOS/UL6^{S309L} is similar to the response to wild-type KOS. DTH response to KOS/UL6^{S309L}. (Left panel) C.AL-20 mice were infected in the right eye with either HSV-1 KOS or KOS/UL6^{S309L} (4 × 10^6 PFU), and, 5 days later, individual groups of infected mice were challenged in the left footpad with 5 × 10^7 PFU UV-inactivated HSV-1 KOS (white bars) or KOS/UL6^{S309L} (black bars). The right and left footpads of each mouse were measured 24 hr later, and the data are given as average specific swelling calculated by subtracting the width of the control right footpad of four mice per group. (Right panel) In vitro proliferation to KOS/UL6^{S309L}. The right superficial cervical draining lymph nodes of C.AL-20 mice were harvested 15 days after ocular infection of the right eye with 4 × 10^6 PFU HSV-1 KOS or KOS/UL6^{S309L}. Cells from these lymph nodes were cultured with syngeneic BALB/c irradiated spleen cells in the presence of UV-inactivated HSV-1 KOS (white bars) or KOS/UL6^{S309L} (black bars). Results are shown as cpm of incorporated [^{3}H]thymidine that was added to each well during the last 16 hr of culture.

tor recombination after cotransfection of Vero cells with a linear S309L DNA fragment and infectious nonreplicating KOS/UL6^m DNA (containing a stop codon in the UL6 gene) (Figure 1A). Recombination between linear S309L DNA and purified KOS/UL6^m DNA allows viral replication and functional selection of recombinants containing repaired UL6 genes. Replication-competent viral isolates (48) were subcloned after cotransfection, and all contained the S309L mutation according to PCR amplification and HindIII digestion (Figure 1A). Sequencing of the PCR product containing the epitope region was performed to confirm conversion of AGC to CTT in two isolates used for further study.

Characterization of the T Cell Response to Mutant Virus KOS/UL6^{S309L}

KOS/UL6^{S309L} grew to titers that were at least as high as wild-type (wt) KOS, and the replication rate of KOS/UL6^{S309L} was indistinguishable from KOS wt after infection of Vero cells in vitro or murine cornea in vivo (Figure 1B). The effect of this amino acid exchange on the ability of the mutant virus to interact with T cells was also measured. T cells from lymph nodes draining HSV-1 KOS- or HSV-1 KOS/UL6^{S309L}-infected corneas responded equally well to wt KOS or mutant KOS/UL6^{S309L}, according to [^{3}H]thymidine incorporation (Figure 1C). Infection with HSV-1 KOS or KOS/UL6^{S309L} also provoked

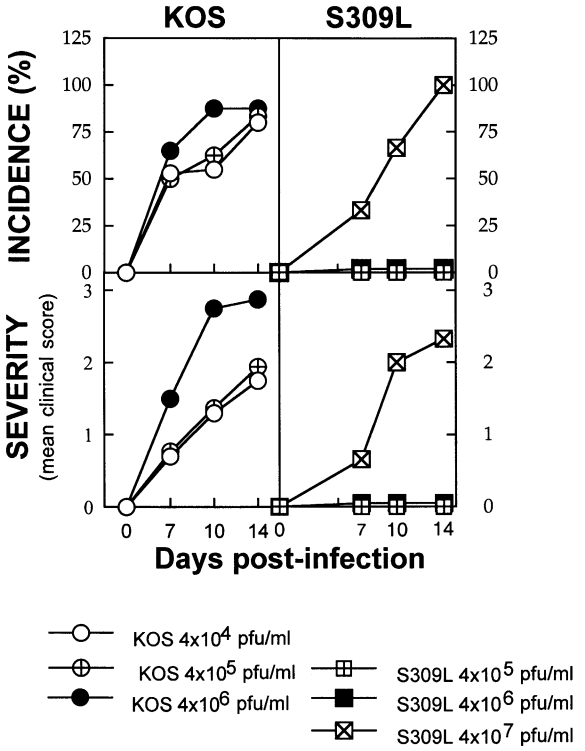


Figure 2. HSK Induction by KOS/UL6^{S309L}

The right eyes of C.AL-20 mice were infected with HSV-1 wild-type KOS at 4×10^4 PFU (open circles), 4×10^5 PFU (plus symbols within circles), or 4×10^6 PFU (closed circles) or KOS/UL6^{S309L} at 4×10^5 PFU (plus symbols within squares), 4×10^6 PFU (closed squares), or 4×10^7 PFU (x within squares), followed by disease assessment on days 0, 7, 10, and 14 after infection. The percentage of each group with detectable disease (i.e., incidence [%]) on days 0, 7, 10, and 14 (minimum score 1) and disease severity shown is based on analysis of HSK in four to eight mice per data point.

similar levels of tuberculin-type delayed-type hypersensitivity (DTH) upon challenge with either UV-inactivated KOS or KOS/UL6^{S309L} (Figure 1C). Since the *UL6* mutation did not inhibit the ability of the virus to replicate, interact with T cells, or induce DTH (cellular) immunity, we were able to ask whether the local inflammatory response provoked by a virus lacking a putative mimic epitope (HSV-1 KOS/UL6^{S309L}) caused HSK.

HSK Induction by KOS/UL6^{S309L}

Corneal infection with 4×10^4 PFU wild-type KOS was sufficient to induce HSK. However, KOS/UL6^{S309L} did not induce detectable HSK in concentrations less than 4×10^7 PFU (Figure 2). Thus, an amino acid exchange that affected the UL6 T cell epitope but not other viral functions decreased the efficiency of disease induction by approximately 10^3-fold compared to wild-type KOS virus. Histologic analysis of corneas from mice infected with 4×10^6 PFU KOS wt or KOS/UL6^{S309L} revealed that both viruses provoked similar levels of inflammatory response 5 days after infection (Figure 3A) during the period of active HSV-1 expression (the virus is no longer detectable in the cornea at the RNA or protein level after day 6) (Daheshia et al., 1997). The KOS/UL6^{S309L}-induced

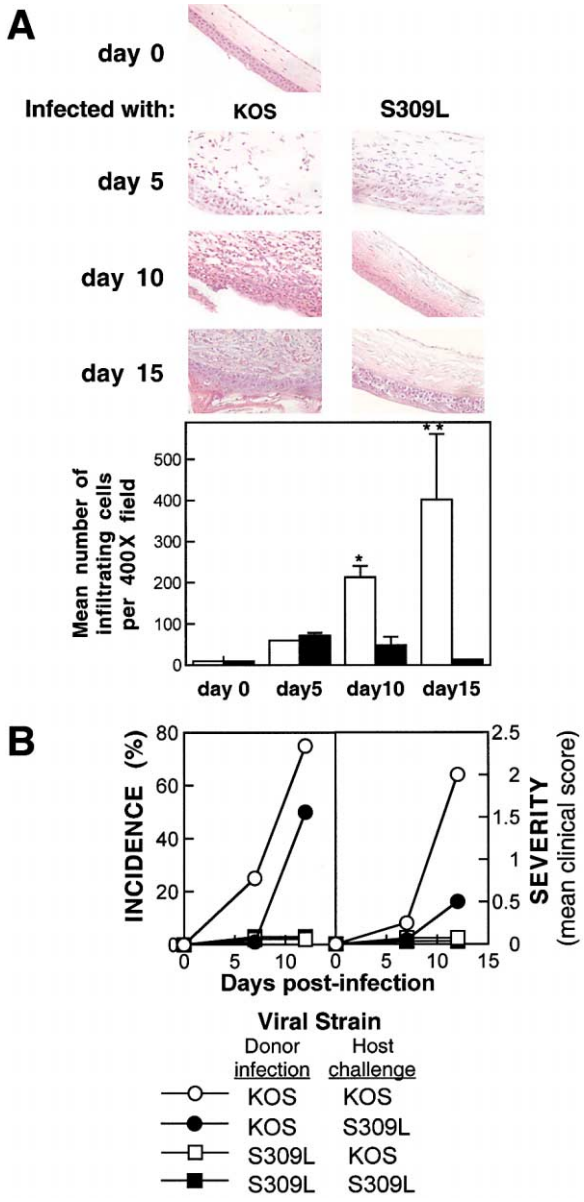


Figure 3. HSK Induction by the KOS/UL6^{S309L} Mutant

(A) Histologic analysis of the inflammatory response of C.AL-20 mice. After ocular infection by HSV-1/KOS (white bars) and S309L mutant (black bars); infected eyes were fixed at the indicated days after infection, sectioned, and stained with hematoxylin-eosin. Infiltrating cells per field (400×) were counted (standard error shown). Numbers indicate average of five fields. *, $p = 3 \times 10^{-5}$; **, $p = 0.008$.

(B) Adoptive transfer of HSK by CD4 cells from mice infected with KOS or KOS/UL6^{S309L}. The right eyes of C.AL-20 mice were infected with 4×10^6 PFU/eye of wild-type KOS or the KOS/UL6^{S309L} mutant virus. After infection (7 days), CD4$^+$ T cells were isolated (as described in Experimental Procedures) from the draining lymph nodes of these mice, and 10^6 CD4 cells from KOS-infected (circles) or KOS/UL6^{S309L}-infected (squares) mice were transferred intravenously into syngeneic BALB/c-*RAG2*$^{-/-}$ mice. After T cell transfer (2 days), recipient mice were ocularly infected with 4×10^5 PFU/eye of either KOS (open circles, open squares) or KOS/UL6^{S309L} (closed circles, closed squares) virus and scored for disease on days 7 and 12 as described in Experimental Procedures. Each point represents at least six mice.

inflammatory response diminished and resolved over the next 10 days, while the KOS-induced inflammatory response progressively increased during this time in the absence of detectable HSV-1.

Adoptive Transfer of HSK by CD4 Cells from KOS/UL6^{S309L}-Infected Mice

We then defined the ability of the S309L mutant virus to stimulate pathogenic CD4 cells according to transfer of HSK. CD4 cells from donors infected with KOS/UL6^{S309L} were unable to transfer detectable HSK to *RAG-2*$^{-/-}$ recipients challenged with wt KOS in contrast to CD4 cells from donors infected with wt KOS, which reproducibly transferred robust HSK into *RAG-2*$^{-/-}$ recipients upon infection with HSV-1 KOS (Figure 3B). The 10^3-fold reduction in HSK activity of the KOS/UL6^{S309L} virus and its failure to induce pathogenic CD4 cells indicate that the UL6 viral mimic critically contributes to activation and expansion of T cells that initiate this autoimmune disease.

Initiation of HSK by CD4 Cells Bearing the C1-6 TCR

These findings suggest that CD4 T cells that express a TCR that recognizes the UL6 mimic and a corneal autoantigen can initiate HSK after viral infection. We have previously defined a $V_\beta8^+V_\alpha11^+CD4^+$ T cell clone (C1-6) that expressed this pattern of crossreactivity and transferred HSK into syngeneic *RAG-2*$^{-/-}$ hosts (Zhao et al., 1998). We therefore expressed the C1-6 or control DO11.10 TCR (which recognizes an OVA-derived peptide) transgene in BALB/c-*RAG2*$^{-/-}$ mice to generate "monoclonal" mice that do not contain other T cell clones because of a blocked endogenous TCR recombination (Chen et al., 1993a, 1993b) to directly test the contribution of this TCR to disease.

T cells that develop in BALB/c-*RAG2*$^{-/-}$ C1-6 TCR Tg mice are composed entirely of $V_\beta8^+CD4^+$ T cells (as predicted from the class II reactivity of the C1-6 TCR) (data not shown), and ocular infection by 10^3 PFU HSV-1 KOS provoked HSK in all BALB/c-*RAG-2*$^{-/-}$ C1-6 mice. In contrast, a 10^4-fold increase in viral titer to 10^7 PFU (the highest titer technically feasible) failed to induce detectable HSK in BALB/c-*RAG-2*$^{-/-}$ DO11.10 mice (Table 1A). Histologic analysis of the inflammatory response at day 10 showed a substantial mononuclear cell infiltration of the corneas of BALB/c-*RAG-2*$^{-/-}$ C1-6 mice (69.8 $\pm$ 13/hpf) but virtually none in the corneas of BALB/c-*RAG-2*$^{-/-}$ DO11.10 mice (3 $\pm$ 1.8/hpf).

Increased HSK susceptibility of BALB/c-*RAG-2*$^{-/-}$ C1-6 mice reflected enhanced T cell reactivity because 2×10^4 CD4 cells from BALB/c-*RAG-2*$^{-/-}$ C1-6 mice transferred HSK to naive BALB/c-*RAG-2*$^{-/-}$ hosts, while 2×10^6 CD4 cells from nontransgenic BALB/c mice were required for efficient transfer of HSK, and as many as 2×10^7 CD4 cells from BALB/c-*RAG-2*$^{-/-}$ DO11.10 mice failed to transfer detectable disease (Table 1B). These data indicate that small numbers of CD4 cells that express the C1-6 TCR are sufficient to confer disease while much larger numbers of T cells bearing an irrelevant TCR cannot.

We have previously suggested that mouse strains that are resistant to HSK following HSV-1 KOS infection lack the T cell clones that recognize the C1-6 peptide (Avery et al., 1995; Zhao et al., 1998). A direct test of this hypothesis comes from an analysis of HSK induction in the resistant C.B-17 mouse strain that expresses the C1-6 TCR transgene. We find that the C.B-17 C1-6 TCR tg mice develop severe HSK following HSV-1 KOS infection (4×10^5 PFU), while age- and sex-matched control C.B-17 mice do not develop detectable disease (Figure 4A). Thus, insertion of the C1-6 TCR into the T cell repertoire converts the phenotype of C.B-17 mice from resistant to highly susceptible.

Amelioration of HSK Using V_β Antibody or Peptides

One prediction of these data is that purging of CD4 cells containing the pathogenic $V_\beta8^+V_\alpha11^+$ TCR from the T cell repertoire might ameliorate HSK upon viral infection. We found that depletion of $V_\beta8.1/8.2^+$ cells but not $V_\beta6^+$ cells from C.AL-20 mice markedly reduced disease intensity after corneal infection with HSV-1 KOS without affecting the T cell response to HSV-1 (Figures 4B and 4C). Moreover, removal of $V_\beta8^+$ but not $V_\beta6^+$ cells from CD4 cells infused into BALB/c-*RAG2*$^{-/-}$ hosts virtually eliminated their ability to transfer HSK into adoptive hosts (Figure 4B). Depletion of $V_\beta8^+$ CD4 cells did not affect antiviral activity, because *RAG-2*$^{-/-}$ recipients of $V_\beta8$-depleted CD4 cells were fully protected from the lethal HSV-1 encephalitis that routinely follows ocular infection of mice that are deficient in T cells (Altmann and Blyth, 1985). Moreover, T cells from mice treated with $V_\beta8$ antibody displayed unimpaired proliferative responses to HSV-1 in vitro (Figure 4C). These data extend conclusions drawn from analysis of C1-6 TCR transgenic mice concerning the primacy of $V_\beta8^+$ C1-6 TCR in disease induction and suggest new therapeutic approaches based on depletion of a restricted portion of the T cell repertoire.

A second consequence of the view that C1-6 TCR$^+$ CD4 cells play a critical role in disease induction is that engagement of this TCR by a peptide ligand leading to anergy or apoptosis should inhibit disease development in vivo. The peptide SYFMYSKLRVQKS represents a superagonist that efficiently activates the C1-6 T cell clone at concentrations of less than 0.05 μM (Zhao et al., 1998) and, at concentrations greater than 20 μM, induces CD4 cells from C1-6 TCR transgenic mice to undergo anergy and apoptosis (Figures 5A and 5B). We therefore asked whether this ligand might induce resistance to HSK in both C1-6 TCR and in susceptible (nontransgenic) C.AL-20 mice. Intravenous injection of high concentrations of soluble C1-6 (400 μg/mouse) but not a mutant peptide that no longer activates the C1-6 TCR resulted in almost complete resistance to the development of HSK after infection with HSV-1 (Figure 5C).

Role of Antigen-Specific and Inflammatory Stimuli in the Induction of HSK

The finding that *RAG-2*$^{-/-}$ hosts reconstituted with KOS-primed CD4 cells developed mild but significant disease when challenged with KOS/UL6^{S309L} (Figure 3B) opened the possibility that LPS-induced stimulation of innate immunity and APC may induce disease in hosts containing sufficient numbers of autoreactive T cells. To test this hypothesis, we examined a series of mice that

Table 1. Analysis of HSK after Ocular Infection of BALB/c-*RAG-2*$^{-/-}$ Cl-6 Transgenic Mice

A. Mouse Strain	HSV-1 Challenge (PFU/Eye)	Herpes Stromal Keratitis	
		Percent	Severity
C.AL-20	4×10^3	0	0
	4×10^4	100	2.7
	4×10^5	100	3.3
BALB/c-*RAG-2*$^{-/-}$ Cl-6 tg	4×10^2	20	0.5
	4×10^3	100	2.1
	4×10^4	100	3.2
	4×10^5	100	4.0
BALB/c-*RAG-2*$^{-/-}$ DO11.10 tg	4×10^6	0	0
	4×10^7	0	0

B. CD4 Cell Donor	Disease Incidence (%): CD4 Cells Infused into *RAG-2*$^{-/-}$ Hosts			
	2×10^4	2×10^5	2×10^6	2×10^7
BALB/c-*RAG-2*$^{-/-}$ Cl-6 tg	100	100	100	100
BALB/c-*RAG-2*$^{-/-}$ DO11.10 tg	0	0	0	0
BALB/c	0	20	90	100

(A) Mice were infected with HSV-1 KOS in the right eye, and disease was scored on day 10 and 14 after infection, as described in Experimental Procedures. The percentage of each group with detectable disease on day 14 (minimum score 1) and disease severity shown is based on analysis of HSK in four to eight mice per data point. (B) Purified CD4$^+$ cells from BALB/c, BALB/c-*RAG-2*$^{-/-}$ Cl-6 TCR Tg, or BALB/c-*RAG-2*$^{-/-}$ DO11.10 TCR Tg were adoptively transferred into BALB/c-*RAG-2*$^{-/-}$ mice. Recipient mice were infected with 4×10^5 PFU/eye HSV-1 KOS and scored for HSK. The percentage of each group with detectable disease on day 14 (minimum score 1) shown is based on analysis of HSK in five to eight mice per data point.

contained increasing numbers of autoreactive T cells (naive C.AL-20; <KOS-immune C.AL-20; BALB/c-*RAG-2*$^{-/-}$ C1-6; <KOS-immune BALB/c-*RAG-2*$^{-/-}$ C1-6). While inoculation of LPS or infection by the HSV-1 KOS/UL6^{S309L} mutant virus onto scratched corneas fails to cause disease in naive C.AL-20 or BALB/c mice, 60% of KOS-immune C.AL-20 mice developed HSK after inoculation of LPS into the cornea. Moreover, 100% of BALB/c-*RAG-2*$^{-/-}$ C1-6 transgenic mice but not control BALB/c-*RAG-2*$^{-/-}$ DO11.10 mice developed intense keratitis after inoculation of LPS or infection with KOS/UL6^{S309L} (Figures 6A and 6C). Finally, provision of a mild inflammatory stimulus (corneal scratch) induced severe keratitis in BALB/c-*RAG-2*$^{-/-}$ C1-6 mice that had been immunized with UV-inactivated HSV-1 (Figure 6B). These experiments indicate that (1) direct activation of T cells by the molecular mimic is required to induce disease in normal nonprimed animals containing limiting numbers of autoreactive T cells (naive C.AL-20); and (2) innate immune mechanisms are sufficient to trigger disease in animals that contain expanded numbers of C1-6 TCR$^+$ T cells. Moreover, development of intense HSK in BALB/c-*RAG-2*$^{-/-}$ C1-6 but not DO11.10 transgenic mice after LPS-dependent activation of innate immune mechanisms reemphasizes the autoimmune nature of HSK in this model.

Discussion

Microbial infection often precedes the clinical onset of diabetes (Gamble and Taylor, 1973; Gamble, 1980; Nagata and Yoon, 1992) and relapses of multiple sclerosis (Sibley et al., 1985) and can precipitate murine diabetes (Nagata and Yoon, 1992), demyelinating disease (Rodriguez et al., 1987; Dal Canto and Rabinowitz, 1982; Miller et al., 1990), herpes stromal keratitis (Streilein et al., 1997; Zhao et al., 1998), and myocarditis (Bachmaier et al., 1999). However, there is no consensus view of the relative importance of antigen-specific stimuli (microbial mimics) and nonspecific innate immune mechanisms to the genesis of autoimmune disease, in part because both mechanisms are likely to contribute to most autoimmune disorders, while one or the other may play a dominant role under particular circumstances (Cantor, 2000). Our studies delineate the conditions that determine the importance of these two mechanisms to the development of HSK.

Viral Infection and Molecular Mimicry

The HSK system is particularly well-suited for studying the inciting role of viral infection, because stimulation of the immune system by the virus is limited to an acute period of approximately 5–7 days, after which the virus becomes undetectable at the protein and RNA level (Streilein et al., 1997; Figure 1), in contrast to other viral infections that may provoke chronic immune reactions through smoldering infection (e.g., HSV-1 encephalitis). A direct test of a peptide mimic in HSK induced by HSV-1 KOS comes from analyses of a replication-competent KOS point mutant as well as studies of mice that express a TCR specific for this mimic. The S309L amino acid exchange in the HSV-1 KOS UL6 protein alters the predicted UL6 T cell epitope without affecting viral replication, stimulatory activity for T cells, or induction of DTH and is thus equipped to provide an unimpaired inflammatory milieu (Figure 1). Nevertheless, this viral mutant did not cause HSK in susceptible C.AL-20 mice unless viral concentrations were increased 10^3-fold, achieving levels not normally seen in nature (Figure 2). Histologic analysis of the cornea showed that cellular infiltration after KOS/UL6^{S309L} and wt KOS infection was similar at day 5, when virus was present. However, over the next 10 days, when the virus is no longer detectable, cell infiltration initiated by wt virus progressively increased, while the KOS/UL6^{S309L} virus-initiated cellular response was markedly reduced by day 10 and absent at day 15.

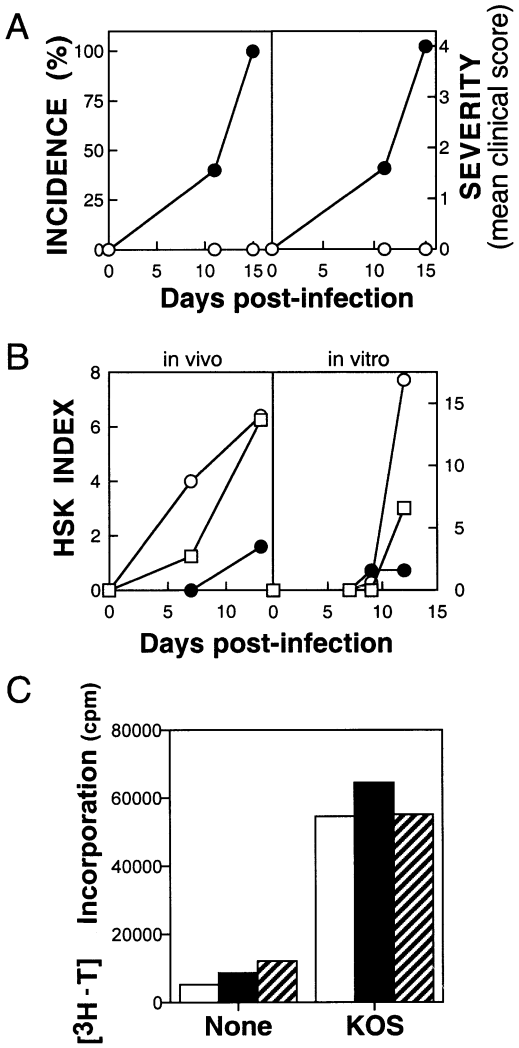


Figure 4. Regulation of HSK Response to HSV-1/KOS by the C1-6 TCR

(A) Effect of C1-6 TCR transgene. C.B-17 (open circles) and C.B-17 C1-6 TCR transgenic (closed circles) mice were infected with HSV-1 (KOS; 4×10^5 PFU). Data shown represent two independent experiments based on analysis of four to five mice per group.

(B and C) Depletion of V_β^+ T cell subpopulation and HSK. (B) (Left panel) C.AL-20 mice were depleted in vivo of $V_\beta6^+$ cells (open circles) and $V_\beta8^+$ cells (closed circles) by i.p. injection of monoclonal antibodies (four doses of 25 μg each), resulting in 98%–99% depletion, or untreated (open squares), before ocular infection with HSV-1 KOS (4×10^6 PFU/eye). (Right panel) Purified $CD4^+$ cells from BALB/c mice were transferred (3×10^6/mouse) into BALB/c-*RAG2*$^{-/-}$ mice after in vitro depletion (99%) of $V_\beta6^+$ cells (open circles) or $V_\beta8^+$ cells (closed circles) or untreated (open squares). All mice were ocularly infected 24 hr later with HSV-1 KOS (4×10^6 PFU/eye) followed by disease assessment as described in Experimental Procedures. HSK index = percent incidence × mean severity of clinical stromal keratitis ÷ 10. Each data point represents the average of at least five mice.

(C) The lymph nodes of HSV-1 KOS-infected, $V_\beta6$-depleted (hatched bars), $V_\beta8$-depleted (black bars), or untreated (white bars) C.AL-20 mice were harvested and cultured with syngeneic C.AL-20 irradiated spleen cells in the presence of UV-inactivated HSV-1 KOS (as described in Experimental Procedures). Results are shown as cpm of incorporated [^{3}H]thymidine that had been added to each well during the last 16 hr of culture.

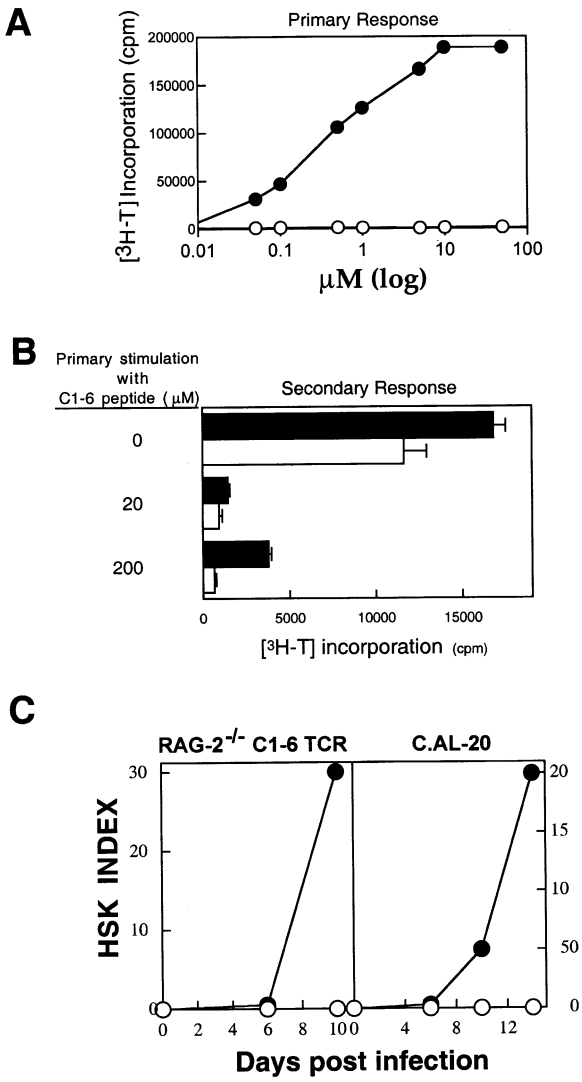
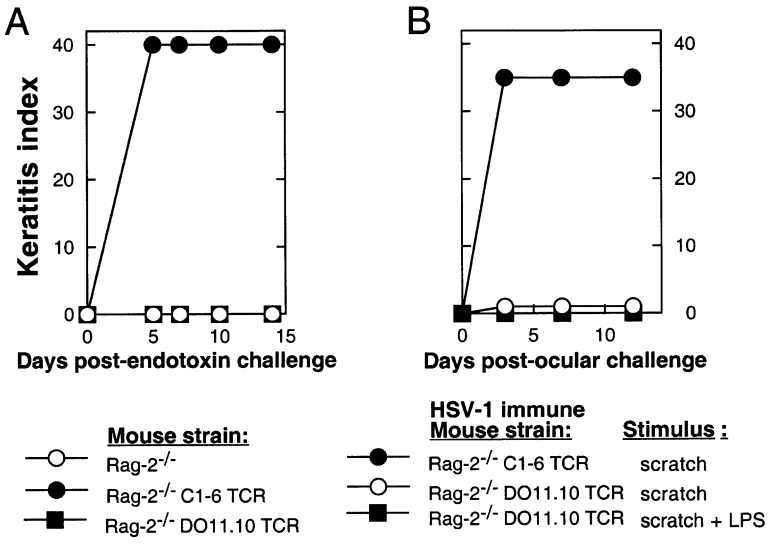


Figure 5. Effect of a Peptide Superagonist on C1-6 T Cell Responses

(A) Stimulation of CD4 cells from BALB/c-*RAG-2*$^{-/-}$ C1-6 mice in vitro. Proliferative response of CD4 cells from BALB/c-*RAG-2*$^{-/-}$ C1-6 mice (10^4/well plus 10^5 irradiated BALB/c splenic cells) to the indicated concentrations of peptide: C1-6 (292-308; SYFMYSK LRVQKS) (closed circles) or K8S mutant (SYFMYSKLRVQSS) (open circles), as described in Experimental Procedures.

(B) Induction of unresponsiveness by C1-6 peptide. After stimulation (5 days) of C1-6 CD4 cells (5×10^4/well) with the indicated concentrations of the C1-6 peptide, cells were restimulated with immobilized anti-CD3 (5 μg/ml; black bars) or C1-6 peptide (100 μM; white bars) and [^{3}H]thymidine incorporation was determined 24–36 hr later. The levels of apoptosis as judged by annexin staining 24 hr after anti-CD3 stimulation of cells primed with 0, 20, and 200 μM peptide were 11, 74, and 82, respectively.

(C) Effect of soluble C1-6 peptide on HSK. BALB/c-*RAG2*$^{-/-}$ C1-6 TCR Tg and C.AL-20 mice were intravenously injected (400 μg/mouse) with either normal C1-6 (open circles) or a mutant C1-6 peptide, K8S (closed circles) twice prior to ocular infection with HSV-1 (4×10^6 PFU/eye), and HSK was scored on the indicated days.



C

Viral challenge (pfu x 10^3/eye)		HSK (%) BALB/c RAG-2 $^{-/-}$ C1-6 Tg	BALB/c RAG-2 $^{-/-}$ DO11.10	C.AL-20
KOS	.4	20	0	0
	4	100	0	0
	40	100	0	100
	400	100	0	100
S309L	.4	20	0	0
	4	100	0	0
	40	100	0	0
	400	100	0	0

Figure 6. Nonspecific Stimuli Induces Keratitis in Mice Containing Large Numbers of Self-Reactive T Cells

(A) The right eyes of BALB/c-*RAG2*$^{-/-}$ (open circles), BALB/c-*RAG2*$^{-/-}$ DO11.10 Tg (closed squares), or BALB/c-*RAG2*$^{-/-}$ C1-6 TCR Tg (closed circles) mice were scratched with a 27 gauge needle, and 16 μg of LPS (Sigma, St. Louis, MO) was added in an 8 μl volume. Clinical keratitis was scored on day 5, 7, 10, and 15. Each point represents at least eight mice.

(B) BALB/c-*RAG2*$^{-/-}$ C1-6 TCR Tg (closed circles) and BALB/c-*RAG2*$^{-/-}$ DO11.10 TCR Tg (open circles, closed squares) mice were immunized three times with 2×10^9 PFU irradiated KOS virus weekly prior to ocular scarification or scarification and LPS (16 μg) treatment. Clinical keratitis was scored on day 3, 7, and 12. Each point represents at least five mice per group.

(C) The indicated strains were infected with increasing titers of HSV-1 (KOS or S309L) in the right eye, and disease was scored on day 10 after infection, as described in the Experimental Procedures. The percent with detectable disease on day 10 (minimum score 1) is shown based on analysis of four to eight mice per group.

This truncated response reflected the inability of KOS/UL6^{S309L} to stimulate disease-inducing T cells; purified CD4$^+$ T cells from the draining lymph nodes of KOS/UL6^{S309L}-infected mice were unable to transfer significant disease to recipient BALB/c-*RAG2*$^{-/-}$ mice compared with the robust activity of CD4 cells from KOS-wt-infected mice (Figure 3B).

Comparative analyses of HSK after HSV-1 infection of *RAG-2*$^{-/-}$ mice expressing the C1-6 and DO11.10 TCR strengthened the conclusion that HSK induction after HSV-1 (KOS) infection depended on activation of a restricted set of CD4 clones. This hypothesis also received support from the finding that insertion of the C1-6 TCR into the T cell repertoire converted the phenotype of C.B-17 mice from resistant to highly susceptible. The hypothesis that the interaction between the C1-6 TCR and the viral mimic played a critical role in disease induction predicted several targeted approaches to immunotherapy of the disease. We found that depletion of V_β8.1/8.2$^+$ cells (the V_β expressed by C1-6) but not V_β6$^+$ cells markedly diminished disease intensity after corneal infection with HSV-1 KOS without affecting the overall antiviral immune response and that intravenous injection of a C1-6 peptide at concentrations that induce apoptosis in vitro ameliorated disease in vivo (Figures 5 and 6).

Viral Infection and Innate Immunity

Understanding the conditions that determine the contribution of innate and adaptive immune mechanisms to the pathogenesis of autoimmunity following infection remains a central and unresolved issue in this field (Horwitz and Sarvetnick, 1999; Medzhitov and Janeway, 1998). The role of nonspecific inflammatory responses is most clearly evident from the finding that tissue-specific expression of cytokine transgenes can provoke autoimmune disease without the need for microbial infection (Horwitz et al., 1997; Akassoglou et al., 1997). In most cases, microbial infection increases the efficiency of T cell activation through enhanced expression of costimulatory molecules, upregulation of MHC expression on professional APC, and attraction of dendritic cells from peripheral to secondary lymphoid tissues (Bachman et al., 1997). Continued activation of innate immunity can also potentiate autoimmune disorders through chronic immune-mediated tissue damage and autoantigen release without the need for specific activation of autoreactive T cells by a microbial mimic (Miller et al., 1990, 1995, 1997; Vanderlugt, 1996; Vanderlugt et al., 1998).

We have compared the relative importance of inflammatory and antigen-specific aspects of infection according to the level of autoreactive T cells in the host. A mild inflammatory stimulus (corneal trauma) is suffi-

cient to provoke disease in animals containing high levels of autoreactive memory T cells (Figure 6C), while a stronger nonspecific stimulus (mutant KOS/UL6^{S309L} virus or LPS) elicits disease in mice that harbor high numbers of naive autoreactive T cells (Figures 6A and 6C). Similarly, MBP1-11 TCR transgenic mice spontaneously develop experimental autoimmune encephalomyelitis (EAE) after exposure to pertussis toxin without specific antigen (Goverman et al., 1993; Linthicum et al., 1982; Munoz et al., 1984). All these observations emphasize that the need for a specific antigenic stimulus is greatest when the host contains limiting numbers of autoreactive T cells, while nonspecific stimuli are sufficient for disease induction in hosts containing expanded numbers of autoimmune T cells. These conclusions are also consistent with observations that Coxsackie B (CB4) virus infection can induce diabetes in BDC2.5^{+} mice provided that that host has developed an expanded population of autoreactive CD4 memory cells (Horwitz et al., 1998; Serreze et al., 2000).

Molecular Mimicry and Autoimmune Disease

Why is expression of a molecular self-mimic essential for efficient disease induction in hosts containing limited numbers of autoreactive T cells? The target self-antigen of HSK is naturally expressed in a nonlymphoid tissue and may not reach levels sufficient for effective cross-presentation to autoreactive T cells, even after microbial infection (Kurts et al., 1998; Ludwig et al., 1999; Sevilla et al., 2000; Serreze et al., 2000). Viruses, on the other hand, are efficiently taken up by professional APC, and peptides derived from them rapidly gain the direct attention of T cells. Presentation of a viral peptide mimic thus bypasses the series of events beginning with release of autoantigen from dying cells and ending with uptake, processing, and cross-presentation by local APC (Ehl et al., 1997; Oxenius et al., 1998). The resulting gain in immunogenicity is reminiscent of the enhanced T cell response achieved after bypassing the need for cross-presentation through expression of tumor antigens in dendritic cells (Klein et al., 2000).

These data open the possibility that subclinical infection by a virus that expresses a mimic can "prime" the host for an autoimmune response upon infection of the target organ by an unrelated virus, thus complicating the search for viral mimics associated with clinical disease. These studies also highlight the importance of testing for viral mimicry early in life (e.g., in genetically predisposed children), since viruses isolated after the onset of clinical disease, such as the dozens identified in MS patients, are likely to contribute to disease pathogenesis through their effects on innate rather than specific immunity. Our studies also indicate that screening for peptide mimics should include assessment of relatively weak microbial ligands. Although the UL6 peptide epitope has a relatively low-affinity interaction with the C1-6 TCR, it is sufficient to induce disease in vivo, while the high-affinity C1-6 peptide (which efficiently stimulates T cells in vitro) inhibits rather than enhances disease in vivo (Figures 4A and 5A). These findings are congruent with the recent observation that MBP-derived peptides that display low-affinity binding to self-MHC efficiently provoke EAE in vivo, while MBP peptide analogs that bind well to self-MHC and strongly stimulate T cells in vitro cause apoptosis of autoreactive T cells and inhibit disease in vivo (Anderton et al., 2001).

Viral Infection and Bystander Damage

In all of the above examples, viral infection through specific or nonspecific mechanisms provokes autoreactive T cells and consequent autoimmune tissue destruction. However, two recent studies by Gangappa and coworkers suggest that a strain of HSV-1 (RE) can induce a nonautoimmune form of keratitis after infection (Gangappa et al., 1998, 2000). In the second study (Gangappa et al., 2000), BALB/c-*RAG2*$^{-/-}$ DO11.10 mice developed keratitis after infection with the HSV-1 RE strain, in apparent contrast to our findings that these mice do not develop HSK after infection by very high titers of HSV-1 KOS (Table 1; Figure 3A). The disparity in these results reflects a difference in the virulence (Thomas and Rouse, 1997) and pathogenicity of the two HSV-1 strains. We have noted that, at relatively high concentrations (4×10^6 PFU), ocular infection by the RE strain can cause clinical keratitis in up to 50% of BALB/c-*RAG2*$^{-/-}$ DO11.10 mice, while infection of the same mice with these titers of HSV-1 KOS fails to induce detectable corneal changes or histological evidence of cellular infiltration. The pathogenicity of HSV-1 RE in BALB/c-*RAG-2*$^{-/-}$ DO11.10 mice may be related to the ability of this strain to attract large numbers of neutrophils (Thomas et al., 1997), in contrast to KOS, which primarily attracts macrophages (Hendricks and Tumpey, 1990), and to its persistence at higher titers in the corneas of immunocompetent mice (Su et al., 1990), leading to attendant damage of the cornea through release of collagenases and/or necrosis of the local vasculature. Alternatively, the intense inflammatory response provoked by the RE virus may allow recognition of low-affinity viral mimics by the DO11.10 TCR.

The development of blindness following clinical herpetic stromal keratitis is marked by intense stromal inflammation leading to irreversible corneal scarring, glaucoma, and cataract. Infection of the mouse strains used here with HSV-1 KOS or RE strains, which model the most destructive and blinding form of this clinical disorder, supports two distinct pathogenic pathways. Infections by relatively nonpathogenic HSV-1 strains such as KOS are translated into an autoimmune attack and blindness through the expression of a viral mimic. A second pathway may result from infection by HSV-1 strains such as RE, which lead to proteolysis and collagen breakdown through nonautoimmune mechanisms that may include bystander damage. Elucidation of the relative roles of these two pathogenic pathways in clinical HSK represents the next step in diagnosis and treatment of a leading cause of human blindness. In a broader context, our studies suggest approaches for evaluation of the relative roles of antigen mimicry and nonspecific inflammation in a variety of clinical syndromes, ranging from bacterial arthritis to some forms of atherosclerosis (Gross et al., 1998; Bachmaier et al., 1999).

Experimental Procedures

Mice

C.AL-20 and C.B-17 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME); DBA/2 and CD1 mice were purchased

from Charles River Laboratory, Inc. (Wilmington, MA); and BALB/c and BALB/c-*RAG2*$^{-/-}$ mice were purchased from Taconic Laboratories, Inc. (Germantown, NY). The OVA-TCR (DO11.10) transgenic mice were crossed into the *RAG2*$^{-/-}$ background (confirmed by PCR and FACS analysis). All mice used for experimentation were 6–8 weeks of age and housed in microisolator cages in the animal biosafety level 2 of the Dana Farber Cancer Institute animal facility.

Construction of KOS/UL6^{S309L} HSV-1 Replication-Competent Mutant Virus

The mutant UL6^{S309L} allele was constructed using the pZZ plasmid (Zhao et al., 1998) containing a 1.3 kb segment of UL6 and the Clonetech Transformer^{TM} Site-directed mutagenesis kit to alter UL6 codon 309 from AGC encoding Ser to CTT encoding for Leu creating plasmid pMES2. These nucleotide changes introduce a HindIII site at bp 922 in the *UL6* coding sequence. The 1 kb MluI-SphI fragment from pMES2 containing the S309L mutation was subcloned into plasmid pMES12, which contains the entire *UL6* coding sequence and a large portion of HSV-1 flanking sequence, to create pMES18.

To integrate the UL6^{S309L} mutation into the HSV-1 KOS genome, 1 μg KOS/UL6^{m} infectious DNA and 5 μg BstBI-AflII-digested pMES18 were cotransfected into 1×10^6 BKK21 cells that had been engineered to express UL6 (Patel et al., 1996). The KOS/UL6^{m} infectious DNA contains a C to T point mutation at nucleotide 136 in the *UL6* coding sequence and does not synthesize UL6 protein (Zhao et al., 1998). Recombination between the BstBI-AflII UL6^{S309L} fragment from pMES18 and the KOS/UL6^{m} infectious DNA introduced the S309L mutation while repairing the UL6^{m} point mutation.

To identify and purify a replication-competent virus that contains the UL6^{S309L} mutation, the transfected G33 cells were harvested 5 days after transfection, virus was isolated by three rounds of freeze thawing, and subsequently used to infect Vero cells. Since KOS/UL6^{m} cannot replicate in Vero cells, only recombinants with a repaired UL6^{m} point mutation could grow. Individual plaques (48) from the Vero cell infection were purified and screened for the presence of the S309L mutation by PCR amplification and digestion with HindIII enzyme. All 48 of the viruses analyzed carried the mutation. Sequencing of an extended region (~500 bp) flanking the 5′ and 3′ boundaries of the recombination sites did not reveal any nucleotide differences compared with the wild-type sequence.

Replication Curve for KOS/UL6^{S309L} and WT KOS

In vitro

To compare the replication rate of the KOS/UL6^{S309L} mutant virus to wild-type KOS, 2×10^5 Vero cells were seeded in wheaton glass vials 4 hr prior to infection with 2×10^6 PFU of virus followed by incubation (1 hr at 37°C) and removal of virus by washing. Cells were resuspended in 1 ml of serum-free DMEM medium, and individual vials were removed at the indicated time points, frozen at −70°C, and prepared by repeated freezing/thawing before assaying for viral PFU/ml by standard methods (Sandstrom et al., 1986).

In vivo

C.AL-20 mice were ocularly infected with 4×10^6 PFU wild-type KOS or KOS/UL6^{S309L}; eyes were harvested on days 2, 3, 4, 5, and 6 and assayed for viral particles as described (Sandstrom et al., 1986).

Ocular Infection and Scoring of HSK

Corneas of mice were scarified using a sterile 27 gauge needle (Avery et al., 1995) before infection with HSV-1 (KOS or KOS/UL6^{S309L}) in the right eye, and disease severity was scored on different days after infection as described (Avery et al., 1995), based on the degree of corneal opacity: ≤25% of cornea, 1; ≤50%, 2; ≤75%, 3; 75%–100%, 4. Corneal opacity represents irreversible and progressive destruction characteristic of necrotizing keratitis resulting from severe stromal edema and necrosis with ulcerations seen on histological sections of the cornea. Neovascularization or corneal clouding were not used to measure stromal keratitis, since these changes may be transient and reversible. Incidence of disease is measured as the percentage of mice with a severity score ≥1. In certain experiments, disease score is summarized as "HSK index" = severity (mean clinical score) × incidence (%) divided by 10.

Histology

Mice were sacrificed on day 5, 10, and 15 postinfection, and infected eyes were enucleated and immediately frozen before tissue sections were stained with hematoxylin-eosin and observed for thickness of cornea and number of infiltrating cells.

Construction of the C1-6 TCR Transgenic Mice

The C1-6 TCR transgenic mouse line was created by cloning the α and β chains from the C1.6 keratogenic T cell clone (Avery et al., 1995). The α-rearranged cDNA (~800 bp) was amplified by anchored PCR as described (Frohman et al., 1988), cloned into SalI-SmaI-digested pSP72 (Promega Corp., Madison, WI) to create α11I, and confirmed by sequence analysis. The XhoI-BglII fragment from α11I containing the complete coding sequence for the α chain was subcloned into the pHSE3′ transgenic vector (Pircher et al., 1989), placing the α chain under the transcriptional control of the H-2K^{b} promoter. Similarly, the β-rearranged cDNA (~930 bp) was amplified by RT-PCR, cloned into SmaI-HindIII-digested pGEM7zf (Promega Corp.) to create BETAI, and sequenced. The XhoI-BamHI fragment from BETAI containing the complete $V_\beta8.1/D_\beta1.1/J_\beta1.4/C_\beta1$ sequence was subcloned into the pHSE3′ vector and placed under the control of the H-2K^{b} promoter. The two XhoI fragments from pHSE3′α and pHSE3′β were used to produce the transgenic mice, as described (Hogan et al., 1986). DBA/2 (H-2^{d}) × BALB/c (H-2^{d}) eggs were comicroinjected with α and β chain constructs, implanted in CD1 females to produce mice with a complete C1-6 TCR. These mice were crossed to BALB/c mice for three to four generations before crossing onto the BALB/c-*RAG2*$^{-/-}$ genetic background. The TCR transgene expression and *RAG2*$^{-/-}$ defect were confirmed by both PCR and FACS analysis.

Delayed-Type Hypersensitivity

C.AL-20 mice were infected in the right eye with HSV-1 KOS (4×10^6 PFU), and, 5 days later, individual groups of infected mice were challenged in the left footpad with 5×10^7 PFU UV-inactivated HSV-1 KOS or KOS/UL6^{S309L} (Foster et al., 1986), and the right and left footpads of each mouse were measured 24 hr later with a Fowler micrometer (Schlesinger Tool, Brooklyn, NY).

In Vitro Proliferation to KOS/UL6^{S309L}

The right superficial cervical draining lymph nodes of C.AL-20 mice were harvested 15 days after ocular infection of the right eye with 4×10^6 PFU HSV-1 KOS or KOS/UL6^{S309L}. Cells from these lymph nodes (1×10^5 per well) were cultured with syngeneic BALB/c irradiated (3000 rad) spleen cells (5×10^5/well) in the presence of 2×10^7 PFU UV-inactivated HSV-1 KOS or KOS/UL6^{S309L}, as described (Zhao et al., 1998). [^{3}H]thymidine (1 μCi) was added to each well during the last 16 hr of culture.

Purification of CD4$^+$ Cells and Analysis by Flow Cytometry

CD4$^+$ T cells were isolated by magnetic negative selection (Dynal, Lake Success, NY) using antibodies to CD8 (53-6.7), B220 (RA3-6B2), Gr-1 (RB6-8C5), and Mac-1 (M1/70) (PharMingen, San Diego, CA). For certain experiments, anti-$V_\beta8$ (F23.1) or anti-$V_\beta6$ (RR4-7) was also used (PharMingen). Cells from lymph nodes or blood were stained using FITC- or PE-conjugated anti-$V_\beta8$(8.1/8.2), anti-$V_\beta6$, anti-$V_\beta4$, anti-$V_\beta2$, and anti-CD4 as previously described (Pestano et al., 1999).

Depletion of T Cells According to V_β Expression

Mice were depleted in vivo of $V_\beta6^+$ cells or $V_\beta8^+$ cells by intraperitoneal injection of monoclonal antibodies (RR4-7 or F23.1; four doses of 25 μg each; day −5, −3, 0, and +2; PharMingen), resulting in 98%–99% depletion (as measured by flow cytometric analysis). Depleted mice were ocularly infected with HSV-1 KOS (4×10^6 PFU/eye). In other experiments, purified CD4$^+$ cells from BALB/c mice were transferred (3×10^6/mouse intravenously) into BALB/c-*RAG2*$^{-/-}$ recipient mice after in vitro depletion by magnetic negative selection (99% by flow cytometry) of $V_\beta6^+$ cells or $V_\beta8^+$ cells.

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