
T CELL RECEPTOR V β GENE EXPRESSION IN EXPERIMENTAL HERPES STROMAL KERATITIS

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SUMMARY

Our study examined T cell receptor (TCR) V β mRNA expression in a murine model of experimental herpes simplex keratitis (HSK). We employed a polymerase chain reaction (PCR) technique to detect TCR V β mRNA expression in the inoculated eyes of both HSK-susceptible and HSK-resistant mice at different time points after corneal inoculation with herpes simplex virus type 1 (HSV-1), followed by Southern blotting and densitometry analysis. In eyes from HSK-susceptible C.AL-20 mice, a more diverse TCR V β transcript usage pattern was detected as compared with that seen in HSK-resistant C.B-17 mice. V β 8 family members were expressed in both strains of mice at days 11, 14 and 21 post-inoculation. By densitometry, at day 11, the intensity of expression of V β 8.2 and V β 8.3 message was significantly greater in the eyes of C.AL-20 mice; V β 8.1 was expressed only in C.B-17 mice. There were obvious differences in the TCR V β expression between HSK-susceptible and HSK-resistant mice. The differences in the intensity of the message expressed by V β 8 family members between the two strains could be correlated to previous experiments that showed V β 8.1,2+ T cells as the main infiltrating cells in the corneas of HSK-susceptible mice by day 11 and 14 after challenge with HSV-1.

Herpetic stromal keratitis (HSK) in mice is a representative model of the blinding corneal disease induced by herpes simplex virus type 1 (HSV-1) in humans. During the past several years, we¹ and others²⁻⁵ have consistently found that immune mechanisms involving T cells play a major role in the pathogenesis of HSK. We have discovered a major

influence of the Igh-1 gene locus on the clinical expression of HSV infection by using Igh-1 disparate BALB/c congenic strains of mice;⁶ C.AL-20 (H-2^d, Igh-1^d) mice develop severe keratopathy, whereas C.B-17 (H-2^d, Igh-1^b) mice are extremely resistant to HSK. The results of experiments performed in several laboratories, including our own, have led to the notion that CD4+ T cells are involved in the induction of HSK, at least in BALB/c mice, and that CD8+ T cells may play a role in protection from HSK.^{7,8}

Studies directed towards the elucidation of whether or not there is a preferential T cell receptor (TCR) V β usage in the keratitis model showed a restricted repertoire of T cells infiltrating the corneas of susceptible mice; immunohistochemical studies showed that V β 8.1,2+ T cells were the main infiltrating cells by day 11 and 14 after challenge with HSV-1, with fewer numbers of V β 3+ and V β 6+ cells seen.⁹ Adoptive transfer of a herpes glycoprotein-D peptide specific, CD4+ V β 8.2+, interleukin 4 (IL-4) producing Th2 clone prior to corneal challenge with HSV-1 accelerated the onset and increased the severity of HSK in susceptible mice, and rendered resistant mice susceptible to HSK.¹⁰ Finally, the *in vivo* depletion of V β 8+ or V β 3+ T cells using monoclonal antibodies reduced the incidence and severity of HSK in susceptible C.AL-20 mice compared with untreated mice.¹

In the present study, we have analysed the TCR V β repertoire in the eyes of C.AL-20 and C.B-17 mice before and after corneal inoculation with HSV-1 in an effort to determine whether HSK-susceptible mice preferentially use a restricted TCR V β repertoire in response to corneal inoculation with HSV and, if so, whether it is a repertoire different from that used by HSK-resistant mice. The TCR V β mRNA expression was analysed by polymerase chain reaction (PCR), followed by Southern blotting and densitometry; such an approach offers

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Table I. Oligonucleotide sequence primers

V β	Sequence	PCR product (bp)
V β 1	TGATTCGAAATGAGACGGTG	458
V β 2	GTGCTGATTACCTGGCCACA	440
V β 3	GAGTGGAGTCAGAAGTCGAT	450
V β 4	CGCTTCCAACCTCAAAGTTC	442
V β 5.1	GAGAGATAAAGGAAACCTGC	310
V β 5.2	AGCAATGTGGTCTGGTACCAGA	580
V β 6	ATGATGCGTCTCGAGAGAAG	436
V β 7	AGATGGTGGGGGTTTCAAGG	448
V β 8.1	GTCCAAGAAGCAAGGTGGCA	613
V β 8.2	TGCTGGCAGCACTGAGAAA	428
V β 8.3	TAGAAACAAGGTGACAGTAACA	614
V β 9	TTCCAATCCAGTCGGCCTAA	436
V β 10	TCTCACCTCTCAGTTCAGAT	439
V β 11	AACGATTCTCAGCTCAGATG	434
V β 12	CGATTCAAAGCTGAGATGCT	432
V β 13	TCTGCTGTGAGGCCTAAAGG	436
V β 14	CTGAACCTCTCAGTTCAG	439
V β 15	CCATCAGTCATCCCAACTTA	432
V β 16	CAGTTGAAAGACCAGATGGT	427
V β 17	GATTCTCAGCTAAGTGTCC	432
V β 18	CAATCAGCCGGCCAAACCTA	436
V β 19	TGTACTTCTGTGCTAGCAGTC	290
C β	TGCACTTGGCAGCGGAAGTG	195

bp, base pairs.

significantly greater sensitivity than the immunohistochemistry techniques used in our earlier studies, although it is important to bear in mind that PCR is detecting mRNA expression rather than protein production. An alternative approach using flow cytometry to analyse V β production was not possible due to the severely limited number of T cells recoverable from infected murine corneas.

METHODS

Mice

Adult 6- to 8-week-old sex-matched C.B-17 (H-2^d, Igh-1^b) and C.AL-20 (H-2^d, Igh-1^d) mice were purchased from the Jackson Laboratories (Bar Harbor, ME). All the mice were handled according to the Animal Care Guidelines from the National Institute of Health and the ARVO resolution on the use of animals in research.

Virus

The HSV-1 (KOS strain) stock was donated by Dr Priscilla Schaeffer (Harvard Medical School, Boston, MA) and was grown in our laboratory as previously described.¹¹ The same virus passage was used in all experiments.

Corneal Challenge

Mice were anaesthetised with 2 mg intraperitoneal ketamine HCl (Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine 400 μ g (Rompun, Mobay, Shawnee, KA). The right cornea of each mouse was scratched eight times in a criss-cross pattern with a 22 gauge needle, and 5 μ l of an HSV-1 suspension containing 2.5×10^5 pfu of virus were instilled in the cul-de-sac, as previously described.¹²

Clinical Scoring

The corneas were examined under binocular microscopy for 21 days after inoculation in order to determine the severity of keratitis. Clinical findings were scored in a masked fashion for the development of stromal oedema and cellular infiltration, corneal neovascularisation, and corneal ulceration, with each parameter graded on a scale of 0 to 4+, as previously described.^{11,12}

PCR Primers and Amplification

Sequence-specific sense primers for 19 V β families and a primer antisense for the constant region of TCR β chain (C β) were synthesised using the phosphoramidite method in an automated DNA synthesiser (Pharmacia LKB, Piscataway, NJ) and purified by using Sephadex NAP-10 columns (Pharmacia). The oligonucleotide primer sequences and the expected sizes of the amplified PCR products are listed in Table I.

RNA Isolation and cDNA Synthesis

Inoculated eyes were harvested from C.B-17 and C.AL-20 mice, both before and at various time points after corneal HSV infection (4, 7, 11, 14 and 21 days post-inoculation). Groups of three mice were used for each time point. Total RNA was extracted using a guanidine thiocyanate/phenol procedure.¹³ Briefly, tissues were homogenised in RNazol (Cinna Biotech Laboratory, Houston, TX) using a Polytron homogeniser (Brinkman Instruments, Westbury, NY). RNA was extracted with chloroform, precipitated with isopropanol, washed with 75% ethanol, and resuspended in DEPC-treated water. The amount of RNA was determined by spectrophotometry. Total RNA was denatured for 10 minutes at 70 °C and incubated at 37 °C for 60 minutes with 0.5 mM oligo dT (20-mer), 5 mM dNTPs (Pharmacia), 20 units of RNasin (Promega, Madison, WI), 20 units of avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer Mannheim, Indianapolis, IN) and 50 mM Tris-HCl pH 8.3 in a total volume of 20 μ l. The reverse transcribed cDNA was diluted 1/10 and stored at -70 °C until use. Specific primers for β -actin, a housekeeping gene expressed at a relatively constant level in all cells, were used to verify the integrity of the synthesised cDNA. In addition, preliminary experiments using the β -actin primers and varying amounts of template cDNA allowed for the selection of an amount of input cDNA for each experimental sample such that subsequent V β PCR amplification remained in the linear or exponential phase for all samples studied.

PCR Amplification

Following the reverse transcription of total RNA into cDNA, PCR was performed by using a wax pellet

Table II. TCR V β transcripts in eyes of C.AL-20 mice (Igh-1^d) and C.B-17 mice (Igh-1^b)

Day	V β																					
	1	2	3	4	5.1	5.2	6	7	8.1	8.2	8.3	9	10	11	12	13	14	15	16	17	18	19
<i>C.AL-20 mice</i>																						
0										+												
4			+			+	+		+	+								+	+			
7	+	+	+				+			+	+	+	+					+	+	+		
11	+	+		+	+		+	+		+	+	+	+	+	+	+	+	+	+			+
14	+	+		+			+	+	+	+	+	+	+	+	+	+	+	+	+			+
21	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+	+			+
<i>C.B-17 mice</i>																						
0			+							+	+							+	+			+
4	+		+						+	+	+							+				
7	+	+		+			+		+	+	+							+	+	+		+
11	+								+	+	+							+				
14									+	+	+											+
21							+	+	+		+						+		+			+

Expression of V β TCR in congenic mice at several time points detected by PCR. '+' indicate presence of V β 1, while blank means absence.

hot-start technique. Briefly, the lower reagent mix of the reverse transcriptase polymerase chain reaction (RT-PCR) contained a final concentration of 12.5 mM Tris-HCl pH 8.3, 62.5 mM KCl (Perkin Elmer, Norwalk, CT), 2.5 mM MgCl₂, 200 μ M of each dNTP (Pharmacia), and 20 pmol of each primer. An AmpliWax PCR Gem 100 (Perkin Elmer) was added to each tube. All the tubes were incubated at 80 °C for 5 minutes and cooled to 4 °C. The upper reagent mix contained a final concentration of 12.5 mM Tris-HCl pH 8.3, 62.5 mM KCl, 2.5 units of AmpliTaq DNA Polymerase (Boehringer) and 10 μ l of cDNA. The total reaction volume was 50 μ l in each tube. The tubes were amplified in a Perkin Elmer 9600 thermal cycler for 30 cycles of 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 2 minutes.

Southern Blot Analysis

PCR products were electrophoresed in 1.5% agarose gels, visualised with ethidium bromide, and blotted onto nylon membranes (Boehringer). The filters were hybridised overnight at 65 °C with a digoxigenin-labelled C β probe internal to all the PCR amplified sequences, then washed with 2 \times SSC/0.01% SDS followed by a final high-stringency wash with 0.5 \times SSC/0.01% SDS. A Fab fragment of anti-digoxigenin antibody (Boehringer) was added followed by washes with 100 mM Tris-HCl and 150 mM NaCl. Membranes were exposed to Kodak XAR-5 film at -70 °C with an intensifying screen.

Scanning Densitometry

Following Southern blotting, scanning densitometry was used to determine the differences in intensity between V β TCR transcripts of the same family members in HSK-susceptible and HSK-resistant mice. The relative intensities of the Southern blot band from each V β amplification were measured in an ImageQuant densitometer (Molecular Dynamics, Sunnydale, CA) and were compared with the C β values for the same sample. The results are expressed as the ratio of the densitometry value for the

particular V β TCR to the densitometry value for the C β TCR.

RESULTS

Stromal keratitis developed in only 7.4% of C.B-17 mice by day 11 and persisted through day 21 post-inoculation, while it was present in 85.2% of C.AL-20 mice by the same day post-infection.

More diverse V β transcripts were seen at the later time points (days 11, 14 and 21) in the inoculated eyes of C.AL-20 susceptible mice as compared with those of C.B-17 mice (Table II). Figs. 1 and 2 are examples of the different V β transcripts found at 11 and 14 days post-inoculation. At day 11 in C.AL-20 mice, V β 2, V β 4, V β 5.1, V β 6, V β 9 to V β 13, V β 15, V β 16 and V β 19 were expressed (Fig. 1); in C.B-17 mice, those V β transcripts were not detected (Table II). At day 14, V β 1, V β 2, V β 4, V β 6, V β 7, V β 9, V β 10, V β 13, V β 14 and V β 16 were expressed in C.AL-20 mice (Fig. 2) but were absent in C.B-17 mice (Table II). At day 21 post-inoculation, V β 1, V β 2, V β 4, V β 5.1, V β 6, V β 7, V β 8.1, V β 9, V β 11, V β 12 and V β 14 were transcribed in C.AL-20 mice but not in C.B-17 mice (Table II).

The expression of V β 8 family members was seen in both strains of mice at days 11, 14 and 21. However, the intensity of expression of V β 8.2 and V β 8.3 was different between these strains of mice at day 11, the time of onset of keratitis. The densitometry readings showed a significantly greater intensity in V β 8.2 and V β 8.3 message in C.AL-20 mice compared with C.B-17 mice (Fig. 3). V β 8.1 was expressed at day 11 only in C.B-17 mice (Fig. 1). At day 14, both strains of mice showed mRNA gene expression of V β 8.1, V β 8.2 and V β 8.3 (Fig. 2), with different but not significantly different densitometer ratios (Fig. 4). The proportion of V β 8.2 message expressed by C.B-17 mice increased from day 11 to day 14 (Figs. 3, 4).

DISCUSSION

The critical role of the cellular immune response in

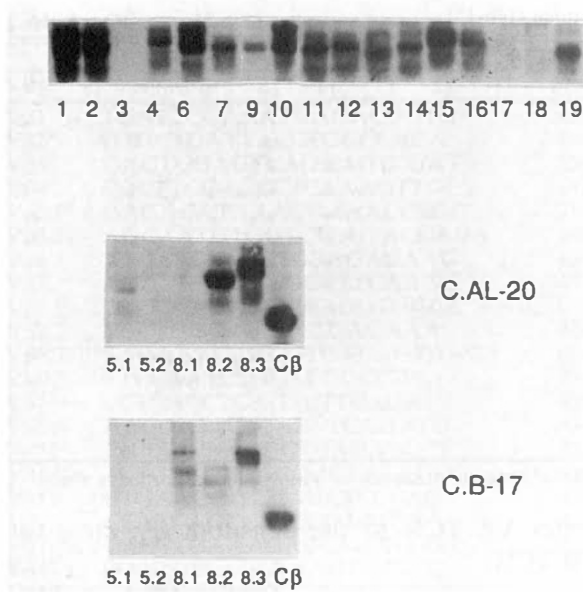


Fig. 1. TCR V β gene expression in eyes of C.AL-20 and C.B-17 mice, 11 days after corneal challenge with HSV. PCR products were detected by ethidium bromide staining followed by Southern blot. V β 1 to V β 19 from C.AL-20 mice are shown, as well as V β 5 and V β 8 family members for each mouse strain.

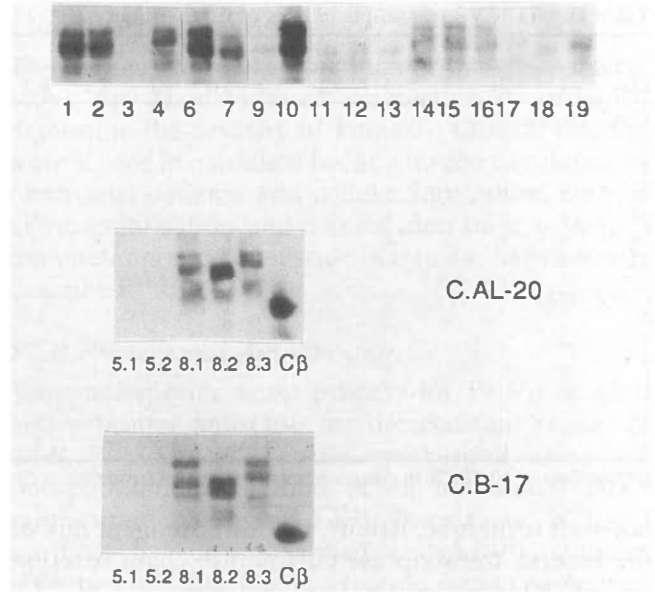


Fig. 2. TCR V β gene expression in eyes of C.AL-20 and C.B-17 mice, 14 days after corneal challenge with HSV. PCR products were detected by ethidium bromide staining followed by Southern blot. V β 1 to V β 19 from C.AL-20 mice are shown, as well as V β 5 and V β 8 family members for each mouse strain.

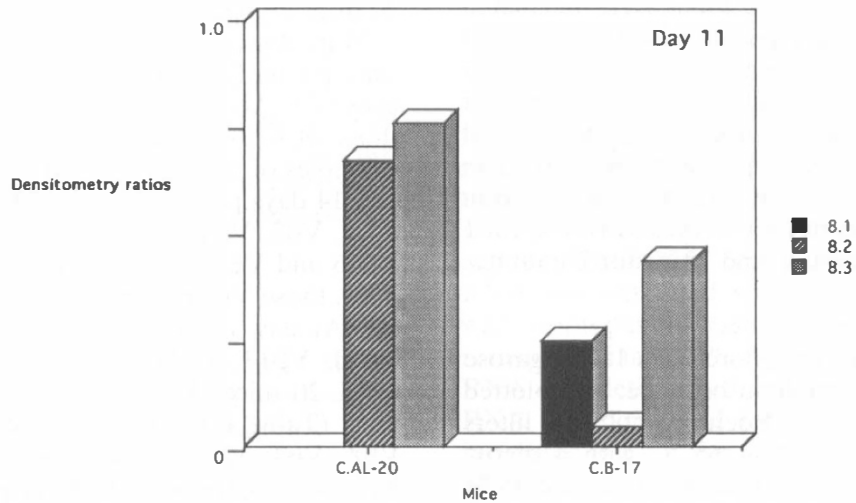


Fig. 3. Autoradiograms were quantified by image analysis as described in Methods. V β /C β ratios of V β 8 family members, obtained from eyes at day 11, are shown.

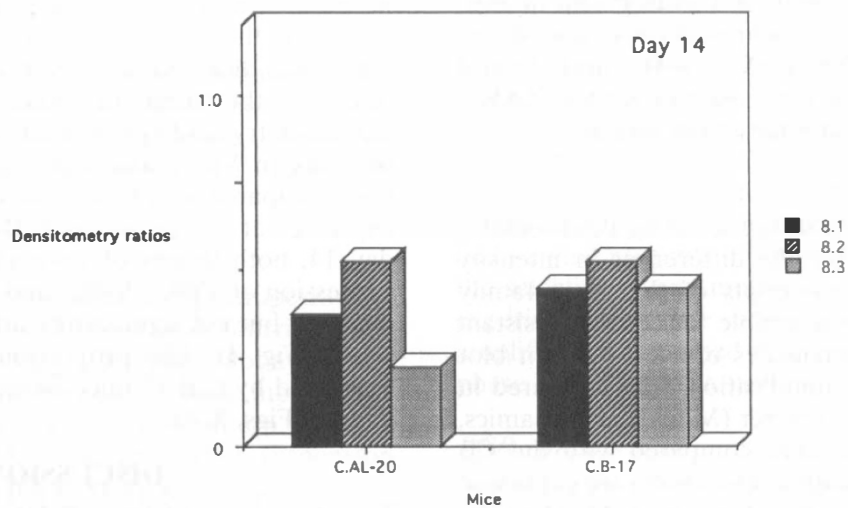


Fig. 4. Autoradiograms were quantified by image analysis as described in Methods

the pathogenesis of HSK has been shown in many studies. Nude mice inoculated in the cornea with herpes virus do not develop keratitis, while their normal littermates do.² Russel *et al.*⁴ showed that the adoptive transfer of non-HSV-specific lymphoid cells into nude mice before HSV-1 corneal inoculation favoured the development of keratitis; HSK was even more severe when HSV-1 immune lymphoid cells were used in the transfer. Attempts to characterise the participation of different subsets of T cells show that the depletion of CD4+ cells by the *in vivo* administration of monoclonal antibodies protects mice from developing keratitis after HSV-1 inoculation, while CD8+ depletion does not,⁷ at least when BALB/c mice are studied. When CD4+ T cells are adoptively transferred into thymectomised mice⁷ or nude mice⁸ from immune donors, herpetic keratitis develops in the recipient mice following HSV inoculation of the cornea.

Effort has been directed towards defining the T cell repertoire in many human and experimental diseases, given the importance of possible immunointervention at that level. However, the interpretation of the data obtained has not been conclusive. Some authors provide evidence for a restricted T cell response in some diseases,¹⁴⁻²¹ while others find significant diversity in the range of TCR gene expression.²²⁻²⁵ In experimental autoimmune encephalomyelitis, restricted TCR V β usage has been shown. Studies have consistently shown that specific monoclonal antibodies and vaccination with specific T cells directed against V β 8 TCR protects animals from demyelinating disease (for review see Vandenberg *et al.*²⁶). We previously reported that T cells bearing V β 8.1,2 TCR, and to a lesser extent V β 3 and V β 6, were the main infiltrating cells into C.AL-20 corneas after HSV inoculation.⁹ It is clear from the results reported herein that these Igh-1 disparate, differentially HSK susceptible congenic mice are not responding to HSV corneal inoculation with different patterns of highly restricted preferential TCR usage, but have different TCR usage kinetics in response to HSV corneal inoculation. In general, more diverse V β transcripts were observed in eyes from HSK-susceptible mice, when compared with HSK-resistant mice, after HSV-1 corneal inoculation. V β 5.1, V β 5.2, V β 7, V β 8.2, V β 8.3, V β 9 and V β 12 were seen in eyes from C.AL-20 mice, but not at any time point in C.B-17 mice.

The diverse V β transcripts found in the eye did not represent a specific V β TCR which could be responsible for the immunopathogenesis of HSK. This may suggest a possible systemic inhibitory factor generated in the resistant mice with subsequently fewer TCR V β transcripts found in eyes, thus avoiding the exuberant inflammatory response to HSV and inhibiting development of HSK, while no

such inhibitory factors are generated in susceptible mice, with the result that more V β transcripts were seen in eyes from HSK-susceptible mice.

Our data clearly show a diverse TCR V β gene expression in this model, in accordance with the results of other authors who did not find a restricted TCR repertoire using different experimental models.²²⁻²⁵ At day 11 in eyes, we found significant differences in V β 8 family members between the Igh-1 disparate congenic strains of mice; increased intensity in the message in V β 8.2 and V β 8.3 was seen in C.AL-20 mice compared with C.B-17 mice. We find this especially interesting in the light of prior work which demonstrated a profound influence on the pathogenesis of HSK by a herpes glycoprotein-D peptide specific CD4+ V β 8.2+, IL-4 producing Th2 clone,¹⁰ and the findings that specific V β 8 T cell subset depletion by using therapy with monoclonal antibodies prevents HSK development in HSK-susceptible mice.¹ The differences that we found in V β 8 usage at the level of mRNA expression further support a role for the V β 8 family members in the pathogenesis of experimental murine HSK. The possible role played by locally produced cytokines in the injured cornea of HSK-susceptible mice in the recruitment of specific T cells with preferential V β usage is currently under study in our laboratory.

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Key words: Herpes simplex keratitis, Polymerase chain reaction, T cell receptor.

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