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ANÁLISIS DEL RECEPTOR Vβ DE LINFOCITOS T EN UN MODELO MURINO DE QUERATITIS HERPÉTICA

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CONTENIDO

RESUMEN

Se utilizaron varios métodos para identificar el repertorio de linfocitos T, con base en la expresión del heterodímero αβ del receptor de linfocitos T (TCR), específicamente la porción variable de la cadena β, que juega un papel en la inmunopatogénesis de la queratitis herpética estromal (QHE) experimental en ratones congénicos de BALB/c susceptibles (C.AL-20 Igh-1^d) y resistentes (C.B-17 Igh-1^b) a la enfermedad. El estudio inmunohistoquímico de las córneas de los ratones susceptibles, demostró que los linfocitos del infiltrado celular expresaron principalmente el TCR Vβ8.1,2 y Vβ3. Mediante la eliminación *in vivo* de linfocitos T Vβ8.1,2 ó Vβ 3 con anticuerpos monoclonales, los ratones susceptibles no desarrollaron QHE a diferencia de los controles. Además, la tranferencia adoptiva de una clona de linfocitos T CD4+Vβ8.2+, específica para un epítope del virus del herpes simplex-1 (VHS-1), la glicoproteína D que contiene los aminoácidos 5-23 (gD 5-23), aceleró el inicio y acentuó la severidad de la QHE en los ratones susceptibles. La detección del RNA mensajero de Vβ por la técnica de la reacción en cadena de la polimerasa (PCR), a partir del material de los ojos de los ratones inoculados en la córnea con VHS-1, reveló una mayor diversidad en la expresión de los mensajeros Vβ en los linfocitos de los ratones susceptibles; once días después de la inoculación del VHS-1 en la córnea se observaron diferencias en los miembros de la subfamilia Vβ8, en los ratones susceptibles y los resistentes.

Estos hallazgos sugieren que los linfocitos T CD4+ con receptor Vβ8.2 son los principales mediadores de la inmunopatogénesis en la QHE.

ABSTRACT

To identify T cell receptor (TCR) Vß expression in a murine model of herpetic stromal keratitis (HSK) induced by herpes simplex virus type 1 (HSV-1), several methodologies were performed. Different BALB/c Igh-1 congenic mice, C.AL-20-Igh-1^{d}-HSK-susceptible, and C.B-17-Igh-1^{b}-HSK-resistant were used. Immunohistochemical staining from corneas of susceptible C.AL-20 mice disclosed Vß8.1,2 CD4 T cells as the prevalent infiltrating cells in the corneas; furthermore Vß3 CD4 T cells were also detected in the cellular infiltrate. Herpetic stromal keratitis was reduced in C.AL-20 mice after *in vivo* Vß8.1,2 or Vß3 T cell depletion, prior to corneal inoculation with HSV-1. Adoptive transfer of T cell clone (CD4+Vß8.2+, HSV-1 glycoprotein gD 5-23 specific) to HSK-susceptible C.AL-20 mice was performed to characterize Vß8.1,2+CD4+T cells as responsible for the destructive keratopathy observed in C.AL-20 mice after corneal inoculation with HSV. Results showed acceleration of HSK onset and the increases in the severity of the disease, compared to the controls. Finally, results of polymerase chain reaction (PCR) to detect TCR Vß mRNA expression, in eyes of C.AL-20 and C.B-17 mice, before and after corneal inoculation with HSV-1, showed higher diversity in Vβ transcripts, in eyes of HSK susceptible mice, when compared with HSK-resistant mice. However, on day 11, significant differences in Vβ8 family members between both strains of congenic mice were found. The participation of CD4+Vβ8.1,2+ T cells play an outstanding role in the immunopathogenesis of experimental herpetic stromal keratitis.

INTRODUCCIÓN Y ANTECEDENTES

La infección de la córnea por el virus del herpes simplex tipo 1 (VHS-1) en el humano, produce una entidad nosológica conocida como queratitis herpética, la cual es una de las principales causas de ceguera en el mundo. En la etiopatogenia de esta enfermedad participan las respuestas inflamatoria e inmunológica desarrolladas en el hospedero, a infecciones recurrentes por el virus. Después del primer contacto con el VHS-1, este permanece latente en el ganglio del trigémino, donde no puede ser atacado por medicamentos antivirales ni por la respuesta inmune del individuo. Ocasionalmente, el virus latente se reactiva, inicia su replicación y se disemina hacia la córnea (1). Se ha reportado la presencia de partículas virales activas en las lágrimas y en la saliva de los pacientes que no desarrollan enfermedad herpética en la córnea (2, 3). Los factores que determinan el establecimiento de la latencia del virus a nivel ganglionar, la reactivación del virus latente y el establecimiento de la enfermedad clínica son muy complejos (2-5). La infección en la córnea esta relacionada principalmente con el VHS-1, sin embargo, el virus del herpes simplex tipo 2 (VHS-2) también puede ocasionar queratitis en menos del 5% de los casos (6). Dada su baja incidencia, en el presente trabajo referiremos primordialmente al VHS-1, primordialmente.

La infección ocular producida por el VHS, en los humanos puede conducir a diferentes formas clínicas de la enfermedad: neonatal primaria, primaria, o recurrente. La enfermedad primaria tanto en el neonato como en el adulto es

infecciosa y ocurre en el hospedero no inmune. La enfermedad recurrente ocurre en sujetos que han tenido contacto previo con el virus y puede ser infecciosa activa, inmune, o ambas (6, 7).

La infección ocular primaria puede manifestarse clínicamente como una blefaritis, una conjuntivitis o una queratoconjuntivitis, acompañadas o no de lesiones en la piel de la región periocular (6, 7).

La enfermedad recurrente debe diferenciarse de una infección ocular herpética primaria que se presenta en un paciente previamente infectado con el VHS en otra parte de su organismo, por ejemplo, una infección herpética localizada en la mucosa oral, de ahi que estos pacientes exhiben una enfermedad similar a las formas recurrentes (1, 6).

La queratitis herpética habitualmente inicia con daño al epitelio corneal manifestándose clínicamente como una úlcera epitelial dendrítica simple o múltiple (queratitis herpética epitelial), la cual puede curar sin dejar una secuela permanente de disminución de la visión (1, 6).

En la queratitis herpética epitelial, la participación de la respuesta inmune no es primordial. Sin embargo, si el VHS-1 invade el estroma corneal por contigüidad, se produce una queratitis estromal herpética, en la cual, la participación de la respuesta inmune es crítica para el desarrollo de la patología corneal (8, 9).

En la forma recurrente del herpes ocular, se establece primero una latencia; a las 48 horas de la infección primaria, el virus viaja por flujo

axoplásmico retrogrado hacia el ganglio sensorial del trigémino, al ganglio ciliar, hacia el núcleo mesencefálico del tronco cerebral y en algunos casos al ganglio simpático, donde permanece en estado latente. El ganglio del trigémino es la principal fuente del virus en cada recurrencia de la enfermedad. El VHS-1 puede llegar al ganglio del trigémino por cualquiera de las tres principales ramas del trigémino: la mandíbular, la maxilar, o la oftálmica. Como consecuencia, una infección inicial orofacial con el VHS-1 puede permitir la latencia del virus y subsecuentemente dar lugar a queratitis herpética recurrente (10).

A pesar de que hay poca o ninguna pérdida visual cuando ocurrre una infección herpética ocular primaria, con cada recurrencia existe una alta posibilidad de inflamación del estroma de la córnea con una subsecuente cicatrización, vascularización, adelgazamiento o perforación corneal, que aumenta el riesgo de daño permanente, por lo que es importante el estudio de la patogénesis de la lesión del estroma corneal, ya que es la causa principal de la ceguera. Por consiguiente, en la patogénesis de la queratitis herpética están implicados factores virales y la respuesta inmune del hospedero, de ahí que se plantee que la enfermedad estromal de la córnea producida por el VHS-1 es el resultado de la respuesta inmune del hospedero, desencadenada contra el VHS-1 (6, 9).

El desarrollo de un modelo experimental para la queratitis herpética ha sido útil para comprender la fisiopatogenia de la enfermedad. La queratitis herpética estromal (QHE) en ratones, producida por el VHS-1, es un modelo experimental

representativo de la enfermedad corneal en humanos. Se conocen las diferencias en la susceptibilidad a las infecciones por el VHS-1 en algunas cepas de ratones, no sólo a nivel de infecciones herpéticas en otros sitios distintos al ojo (11, 12), sino también específicamente para la infección a nivel ocular (13, 14). La susceptibilidad de los ratones a la queratitis herpética está influenciada por los productos de un gen del locus Igh-1 o de un locus estrechamente unido a éste, situado en el cromosoma 12 del ratón (15, 16). De ahi que en el modelo experimental murino de la queratitis herpética, se han estudiado los factores inmunes que participan en la susceptibilidad o resistencia a la enfermedad en diferentes cepas de ratones. Así, los ratones de la cepa C.AL-20 ($H\text{-}2^d$, $Igh\text{-}1^d$) son susceptibles a la queratopatia producida por el VHS-1, mientras que los ratones C.B-17 ($H\text{-}2^d$, $Igh\text{-}1^b$) son altamente resistentes al desarrollo de la misma (16).

Se ha estudiado la participación de la respuesta inmune humoral y se conoce que aunque en una infección primaria o recurrente por el VHS-1 se desencadena una respuesta humoral especifica, ésta aparentemente no es esencial para la recuperación de la enfermedad (17). Por otro lado, se ha encontrado en los ratones infectados, que las IgG´s específicas para el VHS-1 tienen un efecto adverso sobre la replicación viral, (18); otros datos han demostrado que los ratones resistentes producen rápidamente IgG2a e IgG2b anti-VHS y se vuelven susceptibles a la QHE cuando se eliminan sus linfocitos B (19). Sin embargo, se ha establecido que títulos altos de anticuerpos específicos

contra el VHS-1 no protegen contra los episodios recurrentes de queratitis herpética y los títulos de anticuerpos no son modificados por el número de recurrencias de la enfermedad herpética (20), por el contrario, se piensa que los anticuerpos anti-herpes participan en el establecimiento de la latencia del virus en el ganglio del trigémino (21-23).

Otro mecanismo descrito para eliminar a las células infectadas con el VHS-1 es la participación de la citotoxicidad dependiente de anticuerpos (ADCC), la cual está mediada por la interacción de anticuerpos específicos y de células asesinas NK (24).

A pesar de que los anticuerpos evitan la replicación viral no impiden las recurrencias y éstas, junto con los mecanismos inmunes que involucran a los linfocitos T, han demostrado que desempeñan un papel crítico en la patogénesis de la QHE (9, 25-29).

La demostración experimental inicial más elegante sobre la participación de la respuesta inmune celular en la etiopatogénesis de la queratitis herpética estromal fue realizada por Metcalf y cols. (25), quienes utilizaron ratones atímicos; la inoculación del VHS-1 en la córnea de estos animales no produjo queratitis estromal, a diferencia de los ratones eutímicos que presentaron los signos patognomónicos de queratitis, por lo que concluyeron que la participación de los linfocitos T en el desarrollo de la patologia corneal era fundamental. Experimentos posteriores, confirmaron que la transferencia adoptiva de linfocitos T inmunes al

VHS-1 a ratones con inmunidad celular deficiente, les confirió la capacidad de desarrollar QHE, después de la inoculación del VHS-1 en la córnea (26).

El paso siguiente en el estudio de la participación de la inmunidad celular en la patogénesis de la queratitis herpética fue el conocer las subpoblaciones de linfocitos T implicados en ésta; los ensayos de transferencia adoptiva con linfocitos inmunes demostraron que los linfocitos T Lyt-1 participaban en el desarrollo de la enfermedad corneal con el VHS (27). Mediante técnicas de eliminación de subpoblaciones de linfocitos T con anticuerpos monoclonales dirigidos contra linfocitos T CD4+ ó CD8+, se demostró que la eliminación de los CD4+ disminuyó significativamente la susceptibilidad para desarrollar QHE en ratones BALB/c, mientras que la depleción de los CD8+ no presentó efecto sobre la queratitis herpética, y se concluyó que los CD4+ participan en la manifestación de la enfermedad, mientras que los CD8+ están involucrados en la protección de la misma (28), hallazgos que se corroboraron con otras técnicas (26, 29).

Aunque la mayoria de los estudios señalan a los linfocitos T CD4+ como los mediadores de la patologia corneal en la queratitis herpética estromal, Hendricks y cols. (30), reportaron que son los CD8+ y no los CD4+, los responsables del daño corneal.

Una vez comprobada la participación de los linfocitos T CD4+ en la queratitis herpética estromal, se procedió a determinar el mecanismo por el cual actúan; se considera que el daño inicia por la interacción de receptores de los linfocitos con componentes virales expresados en las células infectadas del

hospedero; para entender esta interacción es oportuno recordar aspectos claves de la función de reconocimiento de los linfocitos T.

Una de las funciones más importantes de los linfocitos T es el reconocimiento de péptidos de antígenos en el contexto de las moléculas del complejo mayor de histocompatibilidad (MHC). El punto central para la función del reconocimiento es la expresión, en la membrana celular, del receptor de linfocitos T al antígeno (TCR). Con base en los heterodímeros que expresan sus receptores sobre la membrana celular, existen 2 tipos de linfocitos T: 1) Los linfocitos T que expresan el heterodimero $\gamma\delta$, se encuentran principalmente en los tejidos epiteliales de humanos y de ratones principalmente (31); y 2) Los linfocitos T que expresan el heterodímero $\alpha\beta$ participan en todas las funciones efectoras y reguladoras específicas a un antígeno determinado y responden a los antígenos asociados a las moléculas del complejo mayor de histocompatibilidad clase I (MHC I) o clase II (MHC II), a través de las moléculas accesorias CD8 y CD4, respectivamente (31).

El TCR $\alpha\beta$ tiene 2 cadenas, una es relativamente acídica (cadena α), y la otra es relativamente básica (cadena β) (32, 33). Ambas cadenas son glicoproteínas con un tamaño aproximado de 40 a 60 kDa. y son miembros de la superfamilia de Ig y se encuentran sobre los linfocitos T CD4+ o CD8+ (31).

Los TCR $\alpha\beta$ de los linfocitos CD4+ (cooperadores) o de los CD8+ (citotóxicos), muestran las mismas propiedades bioquímicas básicas. La demostración de que estos heterodímeros regulan el reconocimiento del antígeno

unido al MHC, se logró mediante técnicas de clonación de genes del TCR y de experimentos de transferencia de genes clonados (34).

En la figura 1 se muestra un diagrama esquemático de las cadenas polipeptídicas α y β. Las cadenas polipeptídicas están formadas por regiones variables (V), de diversidad (D), de unión (J) y constantes (C). Estas denominaciones se hicieron comparando las secuencias entre las cadenas homólogas a partir de diferentes clonas de linfocitos T y asumiendo una analogia con las inmunoglobulinas. La porción extracelular del heterodímero αβ, puede considerarse estructuralmente similar al fragmento Fab de una Ig. Las regiones C para cada una de las cadenas α y β del TCR están localizadas en la terminación carboxílo-intracitoplásmica y es la misma para cada uno de los TCR. En el lado amino terminal de las cadenas, correspondiente a la porción extracelular de las mismas, la secuencia de aminoácidos (aa) es diferente para cada TCR, y esta porción es codificada por elementos genéticos V, D, J. Los dominios V tienen estructuras terciarias similares a los dominios V de las Ig´s. El dominio C de la cadena β es similar al dominio C de la Ig; mientras que la estructura terciaria del dominio C de la cadena α del TCR no es de un dominio típico de Ig, puesto que las hebras β, las cuales podrían normalmente formar la parte superior del pliegue β, están muy espaciadas como para formar uniones hidrógeno una con otra. Al igual que los dominios V de las Ig´s, las regiones V de las cadenas α y β del TCR tienen secuencias que son altamente variables de una clona de T a otra, reflejando su papel en el reconocimiento al antígeno (31). El dominio amino

terminal de la cadena β es codificado por un simple exón VDJ formado por el rearreglo de elementos genéticos de la región V, de la región D y de la región J, este patrón de rearreglo genético es similar al de las cadenas pesadas y ligeras de las inmunoglobulinas. Asimismo, las regiones V de las cadenas α y β contienen secuencias que son homólogas a las secuencias Ig codificadas por segmentos genéticos J. La región V de la cadena β también tiene secuencias homólogas a las secuencias de las Ig codificadas por segmentos genéticos de diversidad D (31).

En la porción carboxilo terminal de la cadena α existe una región hidrofóbica que corresponde a la porción transmembranal. Para cada cadena α hay 2 aa cargados: una arginina en el aa 256 y una lisina en el aa 261. Al menos, alguno de esos a.a. es necesario para su expresión en la superficie celular. La cadena α tiene sólo 5 aa en el dominio intracelular y éstos tampoco muestran marcadores de señal intracelular.

Como en la cadena α, en la porción carboxilo terminal de la región constante de la cadena β existe una región hidrofóbica que esta embebida en la porción transmembranal. La región de la membrana tiene la característica poco usual de contener un a.a. de lisina cargado positivamente. Este detalle puede ser importante para comprender la asociación entre el heterodímero αβ y otras cadenas polipeptídicas estructuralmente asociadas sobre la superficie celular. La lisina es requerida para el ensamble y la expresión del TCR sobre la superficie celular. Existen solamente 5 a.a. que forman el dominio citoplásmico, los cuales

no parecen estar involucrados en cualquiera de las vias conocidas de señales intracelulares (31).

El término región variable algunas veces se usa inadecuadamente cuando se refiere al TCR o a las Ig´s. Este término frecuentemente designa al dominio amino terminal entero que contribuye al sitio de combinación, sin embargo, cuando se refiere a los elementos genéticos que codifican el dominio, la región variable designa a un elemento genético, que, con los elementos genéticos de la región de diversidad y de unión, se rearregla para formar el exón que codifica el dominio amino terminal, por lo que su significado se debe determinar con base en el contexto en el cual es usado.

El sitio de combinación del TCR está formado por los dominios amino terminales de las cadenas α y β, de modo similar a los sitios de combinación de los anticuerpos. Los sitios de combinación de los anticuerpos consisten de seis asas como máximo: tres por cada una de las 2 cadenas de Ig. Las porciones que contribuyen a las asas se denominan regiones de determinación de complementaridad (CDRs), y se encuentran en general dentro de las regiones de máxima variabilidad denominadas regiones hipervariables (35, 36).

La comparación de las secuencias de los TCR humanos, de los conejos y de los ratones, demostró que existen substituciones conservadas de aa en las regiones constantes, por lo tanto, los TCR son virtualmente idénticos en las 3 especies (35, 37, 38). Las regiones variables se han diversificado aún mas, pero

el análisis de las secuencias puede predecir que las estructuras son idénticas entre las especies (35, 37, 38).

El heterodímero αβ del TCR le confiere a los linfocitos T la capacidad para reconocer los péptidos antigénicos unidos a moléculas del MHC, sin embargo, la expresión sobre la superficie celular de moléculas del TCR y su función en la activación de los linfocitos T depende de otras 5 proteínas transmembranales que se asocian en forma no covalente con el heterodímero αβ. Esas moléculas en conjunto forman el complejo TCR funcional. Las tres proteínas del complejo se denominan moléculas CD3, e incluyen a miembros de la superfamilia de las Ig´s designadas γ, δ y ε, y un homodímero de cadena ζ unido por puentes disulfuro y que no pertenece a la superfamilia de las Ig´s, o un heterodímero de cadena ζ y cadena γ (Fig. 2). En los ratones, 10 a 20% de los linfocitos T expresan un heterodímero de la cadena ζ y un producto alternativo del gene ζ denominado cadena η. La fórmula estoiquiométrica más común del TCR es: $(\alpha\beta)_2\varepsilon_2\gamma\delta\zeta$-ζ. Los dominios citoplásmicos de las moléculas CD3 presentan de 44 a 81 aa y son capaces de transducir señales hacia el interior de la célula. La porción citoplásmica de cada una de las proteínas γ, δ, y ε del CD3, contiene una copia de una secuencia importante para las funciones de señal, denominada ITAM ("Immunoreceptor Tyrosine-Based Activation Motif") o ARAM ("Antigen Recognition Activation Motif"); la porción citoplásmica de la cadena ζ contiene 3 ITAMs (Fig.2) (31).

En resumen, el reconocimiento del antígeno se debe al heterodímero αβ del TCR solamente y las señales que inician la activación de los linfocitos son transducidas por las proteínas asociadas en el complejo TCR. Además, para iniciar la activación de los linfocitos T, se requiere la presencia de coestimuladores como la molécula CD28 de los linfocitos T, la cual se une a las moléculas B7-1 (CD80) y B7-2 (CD86) expresadas sobre las células presentadoras de antígeno. La molécula CTLA-4 de los linfocitos T también se une a B7-1 y B7-2, pero contrariamente a CD28, la CTLA-4 transmite señales que inhiben la activación de linfocitos T. Otras moléculas de superficie expresadas sobre las células presentadoras de antígeno que actúan como coestimuladoras son VCAM-1 (CD106), ICAM-1 (CD54) y LFA-3 (CD58), las cuales se unen a VLA-4, LFA-1 (CD11a/CD18) y CD2, sobre la superficie de T, respectivamente. Además, hay que considerar con propiedades coestimuladoras a la glicoproteína CD45, así como, a citocinas solubles que regulan el crecimiento y la diferenciación de los linfocitos T (31).

Por lo expuesto anteriormente, se concluye que el TCR tiene una estructura compleja similar a un fragmento Fab de Ig, o un dímero de cadena ligera, con la excepción que ambas cadenas están ancladas en la membrana celular. Ahora bien, estableciendo una relación con el modelo experimental de QHE, cabe mencionar que el locus Igh-1 codifica para la síntesis de la cadena pesada de la IgG2a en ratones y esto pudiera influir en la respuesta de anticuerpos al VHS. A través de interacciones idiotipo-anti-idiotipo, probablemente

el locus Igh-1 regula el repertorio del receptor de linfocitos T, siendo que en el caso de la QHE, los linfocitos T que median la inmunopatogénesis de la enfermedad expresarian los receptores comprometidos en el daño corneal, como se plantea en los artículos que fundamentan esta tesis.

PLANTEAMIENTO DEL PROBLEMA

De acuerdo con los estudios antes referidos que implican la participación de los linfocitos T CD4+ en la inmunopatogénesis de la QHE, el conocer el repertorio de linfocitos T que predomina en la patología corneal, a través de la identificación del heterodímero mas frecuentemente expresado en el TCR de los linfocitos T que participan en la inmunopatogénesis de la QHE, específicamente la cadena β del TCR en su porción variable, se planteó una serie de investigaciones utilizando el modelo experimental de QHE en ratones congénicos BALB/c susceptibles y resistentes a la enfermedad. Para este fin, se utilizaron varias estrategias metodológicas: 1) Tinción inmunohistoquímica de las córneas de los ratones mediante el empleo de anticuerpos monoclonaes dirigidos contra varios Vβ´s del TCR de linfocitos T; 2) Eliminación *in vivo* de subfamilias Vβ del TCR de linfocitos T; 3) Transferencia adoptiva de una clona de linfocitos T (Th2), específica para VHS, la cual expresa el receptor CD4+Vβ8.2+; 4) Utilización de PCR, para estudiar la porción variable de la cadena β de los linfocitos T que participan en el daño corneal.

MURINE HERPES SIMPLEX VIRUS KERATITIS IS ACCENTUATED BY CD4+, $V_{\beta 8.2}$+ Th_2 T CELLS*

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HERPES SIMPLEX VIRUS KERATITIS REMAINS A BLINDING PROBLEM OF major epidemiologic importance to ophthalmologists in all developed countries, despite the development over the past 30 years of antiviral agents effective in the treatment of active episodes of herpes simplex keratitis (HSK). Both viral[1] and host immune response[2-6] factors influence the severity of the inflammatory response that develops in the cornea infected by herpes simplex virus (HSV); it is the unraveling of the details to the immunologic response that has been the most difficult in the research community's attempts to better understand, and hence better treat, this clinical problem.

Study of a murine model of HSK during the past 7 years has helped define the role of the immune response to HSV in both protection from, and creation of, blinding keratitis after HSV inoculation of the cornea.[7-10] At least one gene locus, closely linked to the Igh-1 locus on chromosome 12 of the mouse, tightly regulates the mouse's immunologic response to corneal inoculation with HSV[11] and to anterior chamber inoculation with this virus.[12] Thus, mice which are genetically identical except for a very limited amount of their DNA at and around the Igh-1 locus exhibit dramatically different inflammatory responses to HSV inoculation. Igh-1^a and Igh-1^d mice develop necrotizing stromal keratitis after HSV corneal inoculation, but Igh-1^b mice are highly resistant to such destructive keratopathy; Igh-1^a mice exhibit an intermediate susceptibility to necrotizing HSK.

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The molecular and cellular details of this immunologic response to HSV, which create a complex drama whose finale is either one of protection from or development of pathema, are incompletely understood. An extraordinarily complicated interplay between the components of the natural immune response and the humoral and cellular components of the acquired immune response occurs, regulated by products encoded by a gene located in or around the Igh-1 locus on chromosome 12. The replicating virus is attacked and destroyed in all instances. As the immunologic attack on the virus develops, innocent bystander damage to delicate ocular structures (eg, cornea) critical for good vision hangs in the balance. The mystery is why some mice (Igh-1^b) consistently are spared the vision-limiting keratopathy that other mice (Igh-1^d and Igh-1^c) so routinely develop as a consequence of this inflammatory response.

The cellular and molecular difference between Igh-1 disparate congenic mice that exhibit differential susceptibility to necrotizing HSK are the following:

1. The mice all have equal keratocyte permissivity for supporting HSV replication.
2. Resistant mice make an earlier and more vigorous IgG_{2a} and IgG_{2b} anti-HSV antibody response than do susceptible mice.
3. Resistant mice develop HSK susceptibility if they are treated in such a way that they are rendered B cell-deficient and hence make a deficient anti-HSV antibody response after cornea inoculation with HSV.
4. Susceptible mice are "hyperactive" in their natural killer cell response and in their CD4 T cell response (lymphocyte proliferation assay) to HSV.
5. The keratopathy seen in the susceptible mice is produced by CD4 cells, macrophages, neutrophils, and natural killer cells.
6. The T cell population in the peripheral cornea and in the limbal conjunctiva of susceptible mice is rich in CD4 T lymphocytes and is deficient, in comparison to HSK-resistant mice, in CD8 T cells.
7. Resistant mice have an abundance of CD8 cells in the corneal periphery and in the limbal conjunctiva and few CD4 cells in these areas.
8. In vivo natural killer cell depletion of HSK-susceptible mice converts them into HSK-resistant mice.
9. Passive immunization of HSK-susceptible mice with IgG_{2a} or IgG_{2b} anti-herpes antibody confers complete protection against subsequent development of HSK associated with HSV corneal inoculation.
10. Passive immunization of HSK-susceptible mice with HSV-immune CD8 T cells confers partial protection against subsequent development of HSK after corneal inoculation.
11. Adoptive transfer of T cells into athymic (nude) congenic BALB/c mice, ordinarily resistant to developing HSK, transforms them into HSK-susceptible mice.
12. T cell subset transfers into BALB/c nude mice demonstrates that it is the CD4 T cell subset that is primarily responsible for subsequent development of necrotizing HSK after corneal inoculation.

Still unresolved is the issue of CD8 T cells in their capacity to protect from or participate in the production of HSK in the murine model. Hendricks and Tumpey[13] have reported findings that indicate that CD8 T cells participate in the production of the keratopathy, whereas we[14] and others[15-17] have consistently obtained results through multiple experimental strategies indicating that CD4 T cells (along with NK cells and macrophages), rather than CD8 cells, are the primary cells responsible for the tissue-damaging aspects of the immune response to HSV in the cornea.

The available data suggest that these divergent observations probably relate to the failure of these various investigators to employ identical mouse and herpes virus strains in their experiments, with particular emphasis given to the fact that the mice employed by Hendricks and Tumpey (A/J mice) are defective in that they are deficient in the fifth component of complement. Some of the important things that are not known in this murine model of differential susceptibility to HSK include: (1) What regulates and accounts for the differences in natural killer cell activity between susceptible and resistant strains? (2) Why are Igh-1^d mice hyperactive with their CD4 T cell responses? (3) Why do Igh-1^b mice make a quicker IgG_{2a} andIgG_{2b} anti-HSV antibody response? (4) And, of course, what gene(s) is responsible for this clinical phenomenon of HSK differential susceptibility among Igh-1 disparate congenic mice?

The Igh-1 gene locus encodes for the synthesis of IgG_{2a} immunoglobulin heavy chain. It can, therefore, naturally influence antibody responses to HSV. But the Igh-1 gene locus can also influence T cell responses to HSV. T cell receptor repertoire selection or usage is, at least in some instances, regulated, probably through idiotype-anti-idiotype interactions, by Igh-1 gene-encoded immunoglobulin heavy chain.[18] Therefore, we examined the T cell receptor (TCR) V_β subsets in the corneas of Igh-1 disparate congenic mice exhibiting differential susceptibility to necrotizing stromal HSK, and we employed two strategies to explore the functional relevance of the major V_β TCR infiltrating cell type: (1) in vivo depletion of the V_β TCR subset and (2) creation of a herpes-specific T cell subset expressing than same V_β TCR, with subsequent adoptive transfer of that subset into naive Igh-1 disparate congenic mice prior to HSV corneal inoculation, examining for accentuation or amelioration of subsequent development of HSK. We found that CD4+, $V_{\beta 8}$+ Th_2 T lymphocytes profoundly accentuate the necrotizing stromal

keratitis associated with HSV inoculation into the corneas of HSK-susceptible mice and, further, confer HSK susceptibility onto ordinarily HSK-resistant mice.

METHODS AND MATERIALS

ANIMALS

Inbred BALB/cByJ (H-2^d, Igh-1^a) congenic Igh-1 disparate 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) and were housed and fed in double-barrier isolation in a VR1 laminar flow housing system (Lab products Inc, Biomedic Corp, Rochelle, NJ), with animal care in use conforming to the ARVO guidelines on the Use of Animals in Ophthalmic and Vision Research and the *NIH Guide for the Care and Use of Laboratory Animals*. Six- to 8-week-old mice were sex-matched and used in all experiments.

VIRUS

The HSV 1 (KOS strain) stock was obtained from Dr David Knipe (Harvard Medical School, Boston), and was grown in our laboratory, passed twice in Vero cell monolayers (American Type Culture Collection, ATCC, CCL 81, Rockville, MD), as previously described.[19] The same virus suspended in Eagle's Minimum Essential Medium (MEM) was used in all experiments.

INOCULATION

Mice were anesthetized with 2 mg of intraperitoneal ketamine hydrochloride (Ketalar, Parke-Davis, Morris Plains, NJ) and 400 µg of xylazine (Rompun, Mobay, Shawnee, KS). The right cornea of each mouse was scratched eight times in a crisscross pattern with a 25-gauge needle, and 5 µl of HSV 1 suspension containing 10^5 plaque-forming units (PFU) was instilled in the cul-de-sac, as previously described.[11]

CLINICAL SCORING

Inoculated eyes were observed biomicroscopically with an operating microscope for 2 weeks after inoculation. Clinical findings were scored in masked fashion for development of stromal edema and cellular infiltration, corneal neovascularization, corneal ulceration, with grading of each parameter on a scale of 0 to 4+, as previously described.[11,19]

IMMUNOPATHOLOGY

Mice were killed with CO_2 prior to corneal infection or by days 2, 4, 7, 11, and 14 after corneal challenge. The inoculated eyes were enucleated, snap-frozen, and embedded in Tissue-Tek OCT compound (Ames, Division of Miles Laboratory, Elkhart, IN), and 4-µm sections were prepared (Cryostate microtome, Reichert-Jung, Germany). Sections were fixed to gelatin-coated 12-well microscope slides, air-dried for 30 minutes, and fixed in acetone for 10 minutes. Nonspecific staining was blocked with serum (1:20 in phosphate-buffered saline [PBS]) species-specific for antibodies to be applied, and the sections were then incubated with biotinylated primary antibodies for 30 minutes at 20°C. All antibodies were obtained from Pharmingen, San Diego. Antibodies and dilutions (in PBS) used in the experiments are shown in Table I. After blocking for endogenous peroxidase with 3% hydrogen peroxide in PBS for 10 minutes, sections were incubated with peroxide-conjugated streptavidin (Jackson Immunoresearch Laboratories, Avondale, PA) for 20 minutes at 20°C. The slides were reacted with 3-amino-9-ethyl-carbazole and H_2O_2 in 0.1 M Na-acetate buffer fixed in 4% formalin in 0.1 M acetate buffer counterstained with Gill's No. 3 hematoxylin (Fisher Scientific, Orangeburg, NY) and coverslipped with Gelvatol (Monsanto, Springfield, MA). Negative controls without primary antibodies were analyzed as well.

Control stainings were performed with ascites containing hamster anti-mouse $V_{\beta 3}$ (clone KJ25a, kindly provided by A. Pullen) or rat anti-mouse $V_{\beta 1.2}$ (clone KJ15, kindly provided by J. Kappler) that was purified using protein G columns (Pharmacia, Piscataway, NJ). The incubation (dilution 1:20) was followed by secondary biotinylated donkey anti-hamster or donkey anti-rat antibodies (Jackson Labs), respectively, and peroxidase-streptavidin as already described.

TABLE I: ANTIBODIES FOR IMMUNOHISTOCHEMISTRY STUDY

Primary antibody (dilution)
Rat anti-mouse Thy 1.2 (1:20)
Rat anti-mouse Lyt 2 (CD8, 1:10)
Rat anti-mouse L_3T_4 (CD4, 1:10)
Hamster anti-mouse $V_{\beta 3}$ (1:10)
Mouse anti-mouse $V_{\beta 3.1.2}$ (1:10)
Rat anti-mouse $V_{\beta 6}$ (1:10)
Rat anti-mouse $V_{\beta 7}$ (1:10)
Mouse anti-mouse $V_{\beta 8.1.2}$ (1:20)
Mouse anti-mouse $V_{\beta 8}$ (1:10)
Rat anti-mouse $V_{\beta 11}$ (1:10)
Mouse anti-mouse $V_{\beta 17}$ (1:10)

The conjunctiva, limbus, and cornea were analyzed for stained cells (Zeiss photomicroscopie III, Oberkochem, Germany). Cells were counted on three separate high-power fields (×100 magnification) from two serial sections.

PREPARATION OF CLONED CD4 $V_{\beta 8.2}$ T CELLS

A major epitope region from HSV glycoprotein D was synthesized by Biosearch, San Rafael, CA. This gD peptide contained, by our specifications, amino acids 5-23. We previously used this peptide in immunization experiments and demonstrated that active immunization with this peptide could produce a significant immune response in the immunized animals capable of conferring protection against subsequent herpes infection.[20] HSK-susceptible C.AL-20 mice were immunized subcutaneously in two sites on the flank with a total of 5×10^7 gD 5-23-coupled syngeneic spleen cells. The syngeneic spleen cells (1.4×10^8) were coupled with 20 mg of peptide using the method of Miller and associates.[21] The mice were "boosted" with 500 U of recombinant human interleukin 2 (Genzyme Corporation, Boston, MA) intravenously at the time of immunization. Regional draining lymph nodes harvested 1 week later were stimulated with x-irradiated BALB/c spleen cells and antigen (gD 5-23) in RPMI-1640 media containing penicillin, L-glutamine, nonessential amino acids, sodium pyruvate, 2-mercaptoethanol, and 5% fetal calf serum (FCS) as described previously.[22] Ten days later, T cells were enriched over Ficoll gradients and stimulated with antigen and x-irradiated spleen cells once more. PMA-stimulated EL-4 supernatant[23] was added as a source of T cell growth factors. The T cells were cloned by limiting dilutions in the presence of antigen. Individual clones were expanded in growth factor containing media and tested for antigen specificity by lymphocyte proliferation assay. T cell proliferation was performed in the usual way with assessment of thymidine incorporation.[22]

IN VIVO DEPLETION OF $V_{\beta 8.1,2}$+ OR $V_{\beta 3}$ CD4+ T CELLS

C.AL-20 and BALB/c mice were injected intraperitoneally with 150 μg (total protein) of monoclonal anti-$V_{\beta 8.1,2}$ or anti-$V_{\beta 3}$ in 200 μl PBS, 48 and 24 hours prior to inoculation of the cornea with HSV; additional intraperitoneal injections of 150 μg of antibody were administered 3, 7, and 12 days following corneal inoculation with HSV. Flow cytometric analysis was performed on monoclonal antibody-treated and normal (untreated) mice by harvesting their spleens 14 days after HSV corneal inoculation, separating T cells from the splenocytes by negative selection of all B cells and adherent cells through T cell enrichment columns (Biotex, Alberta, Canada), and T cell labeling with antibodies specific for various V_β glycoproteins. Briefly,

columns were washed with 15 ml Hank's balanced salt solution (HBSS) buffer with 2% FCS, and then activated with polyclonal goat anti-mouse IgG reconstituted in 1.5 ml HBSS and 2% FCS. Unbound polyclonal goat anti-mouse IgG was eluted with 20 ml HBSS and FCS. The column flow rate was calibrated to 1 drop per 6 seconds. The eluted T cells were counted with a hemocytometer, washed, and stained with fluoresceinated (FITC) monoclonal anti-V_β antibodies (Pharmigen, San Diego). The cells were washed with PBS with 0.1% azide, thrice fixed in 2% paraformaldehyde in PBS, and analyzed with a FACScan (Becton Dickinson, FACS systems, Sunnyvale, CA). Data analysis was accomplished by Consort-30 software (Becton Dickinson, Mountainview, CA). The frequency of the various V_β + T cells was analyzed on contour graphs based on 10,000 viable cells collected for each sample. The background staining was less than 0.2% in all experiments.

LYMPHOKINE BIOASSAYS

IL-2 and IL-4 were detected by using the HT-2 cell line as previously described.[23] IL-3 was assessed by using FDC-P1 cell lines.[23] The WEHI-279 cell line was used for IFN-gamma analysis and the L-929 cell line was employed for TNF alpha detection.[23]

ADOPTIVE TRANSFER OF $V_{\beta 8.2}$ + CD4 + T CELLS

T cells (5×10^5) from this $V_{\beta 8.2}$ + CD4 + T cell clone were adoptively transferred, either intravenously or intraperitoneally, into C.AL-20 mice, into BALB/c mice, and into C.B-17 mice, five mice receiving the adoptively transferred cells via each route. These transfers were performed 2 hours prior to corneal inoculation with HSV.

STATISTICS

The significance of differences in clinical keratitis scores was analyzed by chi-square analysis.

RESULTS

KERATITIS

The results from these experiments confirmed the observations made in our laboratory over the past 7 years: C.B-17 ($Igh\text{-}1^b$) mice are extraordinarily resistant to development of HSK, with only 7.4% of these mice developing the keratopathy; C.AL-20 ($Igh\text{-}1^d$) mice, on the other hand, are extraordinarily susceptible to developing necrotizing HSK, with 85.2% of the mice

developing profound keratopathy in these experiments ($P < .001$). These data are illustrated in Fig 1.

ANALYSIS OF T CELL POPULATIONS IN THE CORNEA BY IMMUNOHISTOCHEMISTRY

Mononuclear cells invaded the limbal conjunctiva of both mouse strains between days 2 and 4 following HSV challenge. No T cells were found in the corneas of HSK-resistant C.B-17 mice at any time during the experiment, but T cells accumulated in great abundance in the corneas of the susceptible C.AL-20 mice, being greatest in number by days 11 and 14 postinfection (Table II). The CD4:CD8 ratio in the C.AL-20 murine corneas was 3.2:1 at day 11 and 5.4:1 at day 14. Most of these CD4 cells were $V_{\beta 8.1,2}$+ in their TCRs; some $V_{\beta 3}$ was detected in the CD4 T cells infiltrating the corneas. The entire repertoire of V_{β}-expressing T cells was seen in sections of spleen (Fig 2), with the interesting observation that, while BALB/c congenic mice ordinarily have few $V_{\beta 3}$-expressing cells, encounter with HSV results in a significant appearance of these cells in the spleens of C.AL-20 but not C.B-17 mice. The cells were highly immunologically activated, as determined by the presence of the CD25 activation marker. Obviously, TCRs, $V_{\beta 8.1,2}$ or otherwise, were not found in the corneas of the HSK-resistant C.B-17 mice, since no T cells were present in any of the corneas from these mice following HSV inoculation.

IN VIVO DEPLETION OF CD4+ $V_{\beta 8.1,2}$+ AND OF $V_{\beta 3}$+ T CELLS PRIOR TO HSV CORNEAL INOCULATION IN C.AL-20 HSK-SUSCEPTIBLE MICE

Eight C.AL-20 HSK-susceptible mice were rendered CD4+ $V_{\beta 8.1,2}$+ or $V_{\beta 3}$+ T cell deficient through in vivo anti-$V_{\beta 8.1,2}$ or anti-$V_{\beta 3}$ monoclonal antibody therapy. HSV inoculation of the corneas of untreated C.AL-20 mice and $V_{\beta 8.1,2}$ or $V_{\beta 3}$ depleted mice resulted in the expected development of necrotizing HSK in the control (untreated) C.AL-20 mice; 80% of these mice had developed extensive keratopathy by day 12, and the severity of the keratopathy was extreme (Fig 3). Flow cytometry analysis of the V_{β} TCR subsets following monoclonal anti-$V_{\beta 8.1,2}$ therapy showed nearly complete elimination of $V_{\beta 8.1,2}$ TCR-expressing T lymphocytes from the treated mice (Fig 4). Figs 5 and 6 show that these mice, rendered deficient in CD4+ $V_{\beta 8.1,2}$+ T cells, had a dramatic reduction in both the incidence and the severity of keratopathy following HSV corneal inoculation. Anti-$V_{\beta 3}$-treated C.AL-20 mice also had some reduction in HSK (data not shown).

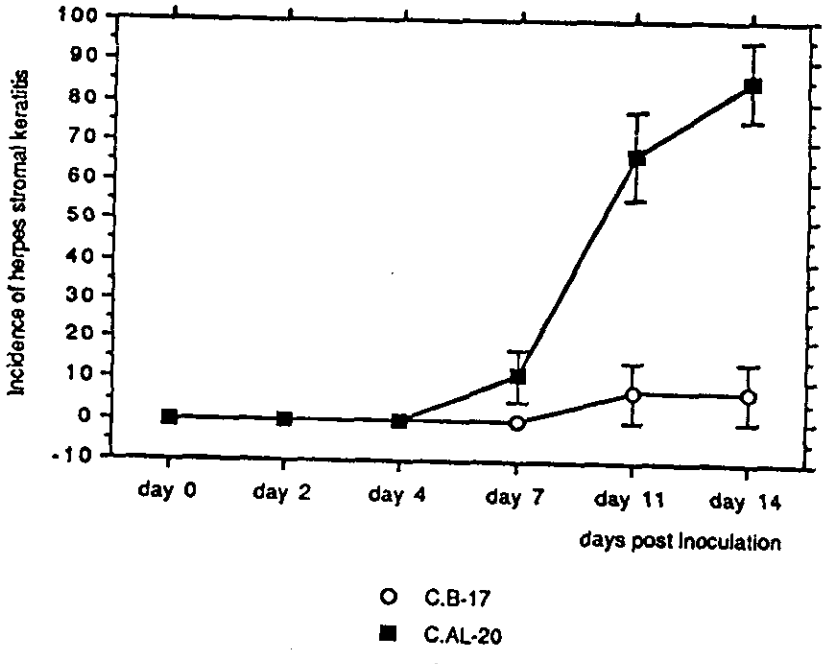


FIGURE 1
Incidence of herpes simplex stromal keratopathy. Note remarkable difference between incidence of keratopathy in HSK-susceptible C.AL-20 mice compared with HSK-resistant C.B-17 mice, very obviously by day 11 and even more so by day 14.

TABLE II: IMMUNOHISTOCHEMICAL ANALYSIS OF MONONUCLEAR CELL SUBSETS IN HSK

GROUPS OF C.AL-20 MICE	Thy1.2+	CD4+	CD8+
Sham immunized			
Conjunctiva	35.8 ± 5.4	2.3 ± 0	1 ± 0.5
Corneal limbus	44.5 ± 11.75	9.75 ± 6	0.75 ± 0.25
Corneal stroma	25.28 ± 4.13	11.7 ± 3.1	1.58 ± 0.92
5-23 gD peptide-KLH, TiterMax			
Conjunctiva	30 ± 10	1.33 ± 0	0.25 ± 0.25
Corneal limbus	4.63 ± 0.13	0.5 ± 0.5	0 ± 0
Corneal stroma	1.58 ± 0.25	0 ± 0	0 ± 0

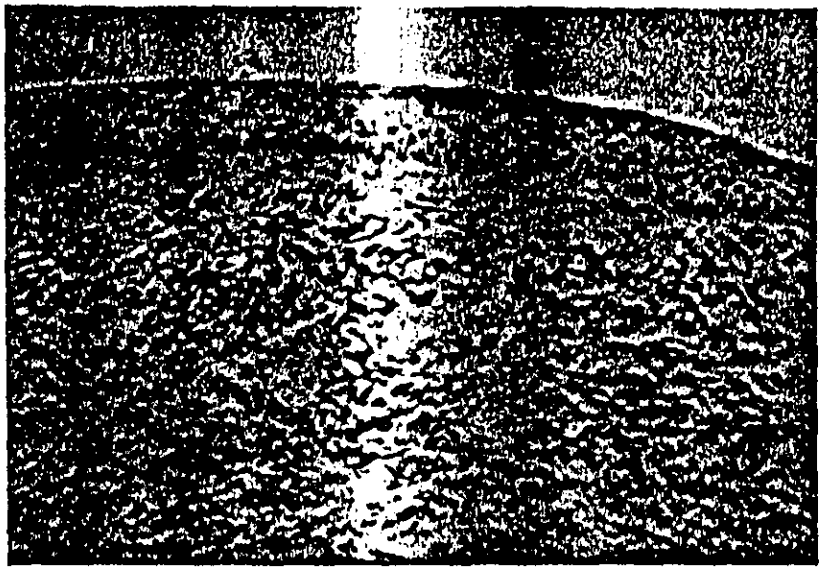


FIGURE 2
C.AL-20 cornea, day 11 following HSV corneal inoculation. Large numbers of NK cells, macrophages, and CD4 T lymphocytes in cornea. All of latter use $V_{\beta 8.1,2}$ glycoprotein in their T cell receptors, as shown by staining of these cells with anti-$V_{\beta 8.1,2}$ antibody employed in this photograph (immunoperoxidase technique, × 100).

ADOPTIVE TRANSFERS OF HSV gD PEPTIDE (5-23)-SPECIFIC CD4+ $V_{\beta 8.2}$+ T CELLS INTO C.AL-20, BALB/c, AND C.B-17 MICE PRIOR TO HSV CORNEAL INOCULATION

A T cell line was derived from lymph node cells of HSK-susceptible C.AL-20 mice immunized with syngeneic spleen cells coupled with HSV-1 gD 5-23 peptide. After 5 months of stimulation with the gD 5-23 peptide, T cells were cloned by limiting dilution, and both the original T cell line (CAL.J) and the ultimate clone derived (C.J. 14) proliferated in response to the gD 5-23 peptide used for immunization and for in vitro selection, and an irrelevant 13 mer HSV-1 glycoprotein C peptide failed to induce proliferation of these cells, incidating the fine specificity of the T cells.

Antigen recognition by the cloned cells (Fig 7) was dependent on CD4, MHC-encoded I-E^d as well as LFA-1 determinants. The cells produced interleukin 4 and used it as their autocrine growth factor, since the addition of anti IL-4 but not anti IL-2 antibody inhibited the proliferation of the clone. Antigen stimulation of the T-cell clone resulted in the release of IL-4 into the culture supernatant as assessed by bioassay. Proliferation of the IL-2/IL-4-dependent HT-2 cell line induced by antigen-activated T cell

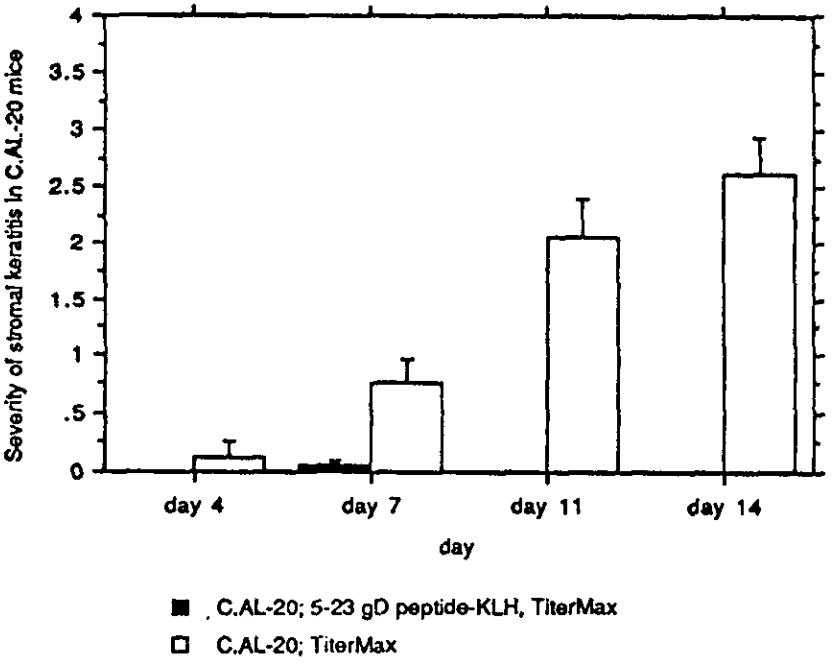
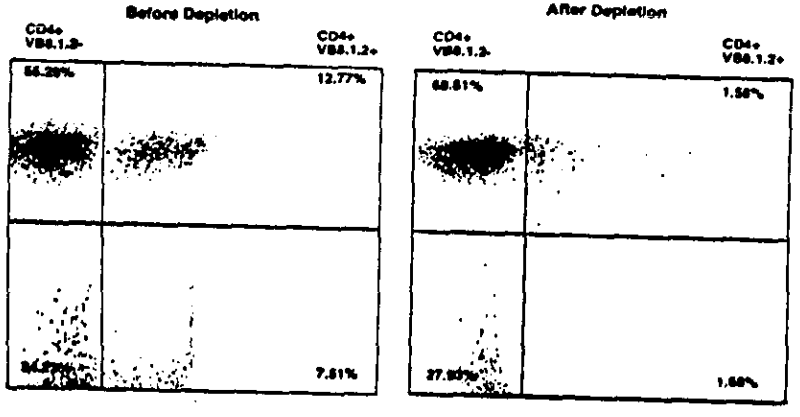


FIGURE 3
HSK severity in C.AL-20 mice. This figure demonstrated that by day 11, severity of herpes keratopathy is quite impressive, at 2+ level on 0-4+ scale and, additionally, that keratopathy can essentially be completely prevented by prior immunization of these HSK-susceptible mice with gD peptide (5-23).

supernatant was blocked by anti IL-4 but not by anti IL-2 antibody. The T cells from this clone produced IL-3 but not the Th_1 characteristic lymphokines, interferon gamma, and tumor necrosis factor alpha. The T cells of this clone proliferated in response to IL-1 like other Th_2 cells reported earlier.[23] Taken together, these characteristics of the cells from our clone are consistent with the phenotype of the Th_2 CD4 murine T cell.[24] Additionally, the cells from our clone reacted with the F23.1 antibody, an antibody specific for the $V_{\beta 8.2}$ protein. Antibody to the CD4 determinant uniformly stained our cells, whereas antibody to the CD8 marker did not stain them. Our cells, therefore, are T cells, specifically CD4 T cells, and CD4 T cells that employ the $V_{\beta 8.2}$ protein in their TCR. These cells have restricted specificity to an epitope in gD of herpes simplex-1.

Adoptive transfer of these cells into HSK-susceptible C.AL-20 mice or into moderately susceptible BALB/c mice resulted in a dramatic change in susceptibility to subsequent HSV corneal inoculation. Intraperitoneal and



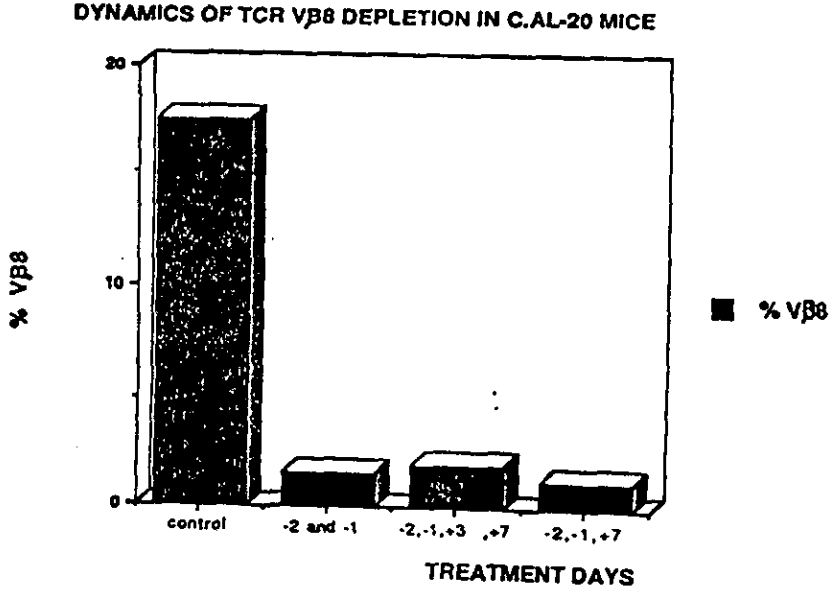


FIGURE 4
Flow cytometry results of both $V_{\beta 8.1,2}$ treated and untreated C.AL-20 mice. Flow cytometry demonstrates that before treatment 11.28% of lymphocytes were CD4+ $V_{\beta 8.1,2}$+ (A) and after therapy only 1.19% of T cells were double positive CD4/$V_{\beta 8.1,2}$ (B). Bar graph (C) demonstrates that this depletion was long-term, sustained.

Intravenous adoptive transfer of 5×10^5 cells resulted in dramatic acceleration of the onset of HSK, with its development as quickly as 2 days after corneal inoculation rather than the typical 8- to 10-day onset of HSK. One hundred percent of the C.AL-20 mice that received the gD-specific T cells developed severe HSK; 60% of the control C.AL-20 mice developed HSK. The severity of the HSK in the C.AL-20 mice that received the adoptively transferred gD-specific T cells was considerably greater than that of the control C.AL-20 mice (Fig 8).

Similar results were seen after adoptive transfer of these cells into naive BALB/c mice, mice which are ordinarily less susceptible to development of severe necrotizing HSK after HSV inoculation, compared with C.AL-20 mice. The onset of keratitis was accelerated, the incidence of keratitis was accentuated, and the severity of the HSK was dramatically increased (Fig 9). Adoptive transfer of these cloned T cells into ordinarily HSK-resistant C.B-17 mice resulted in a biphasic HSK developmental pattern, with moderate HSK at postinfection days 2 to 4, resolution of the HSK, and subsequent re-emergence of mild HSK at days 8 to 12 postinfection. Control

DAY 8 INCIDENCE OF HSK

MICE	INCIDENCE OF HSK
C.B-17	2/10
C.AL-20	8/10
C.AL-20 with antiVβ8 therapy	3/10
C.AL-20 with irrelevant MAB therapy	7/10

FIGURE 5
Day 8 incidence of keratitis. Note that incidence of HSK in normally HSK-susceptible C.AL-20 mice is dramatically reduced with anti-$V_{\beta 8.1,2}$ monoclonal antibody therapy.

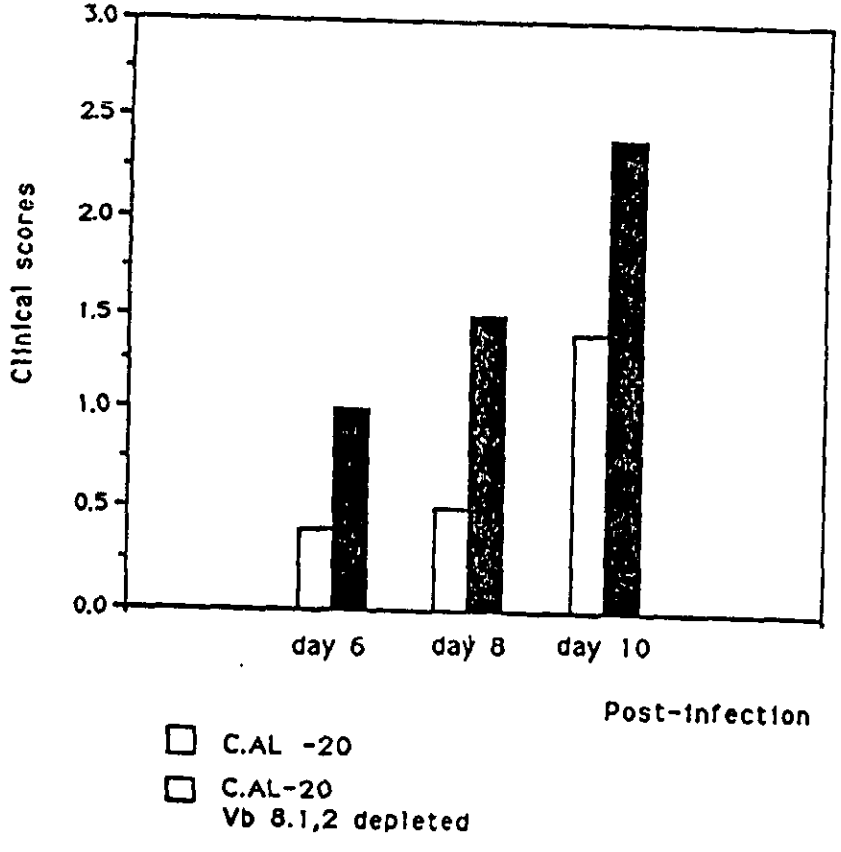


FIGURE 6
HSK severity. Notice reduction in severity of HSK in C.AL-20 mice rendered deficient in CD4+ $V_{\beta 8.1,2}$+ T cells by monoclonal anti-$V_{\beta 8.1,2}$ antibody therapy.

studies, with similar adoptive T cell transfers into these same murine strains, employing a Th-2 clone, which is specific for purified protein derivative, an irrelevant antigen for the purposes of our studies, failed to influence the development of HSK in these mice. Additionally, PPD-specific Th-2 clone cells failed to promote the development of a corneal inflammatory response when such cells were adoptively transferred into C.AL-20 and into BALB/c mice, with subsequent corneal inoculation with purified protein derivative. Taken together, these data indicate that specific recognition of HSV by transferred gD-specific T cells is necessary and sufficient for the accelerated onset and increased severity of HSK in HSK-susceptible mice.

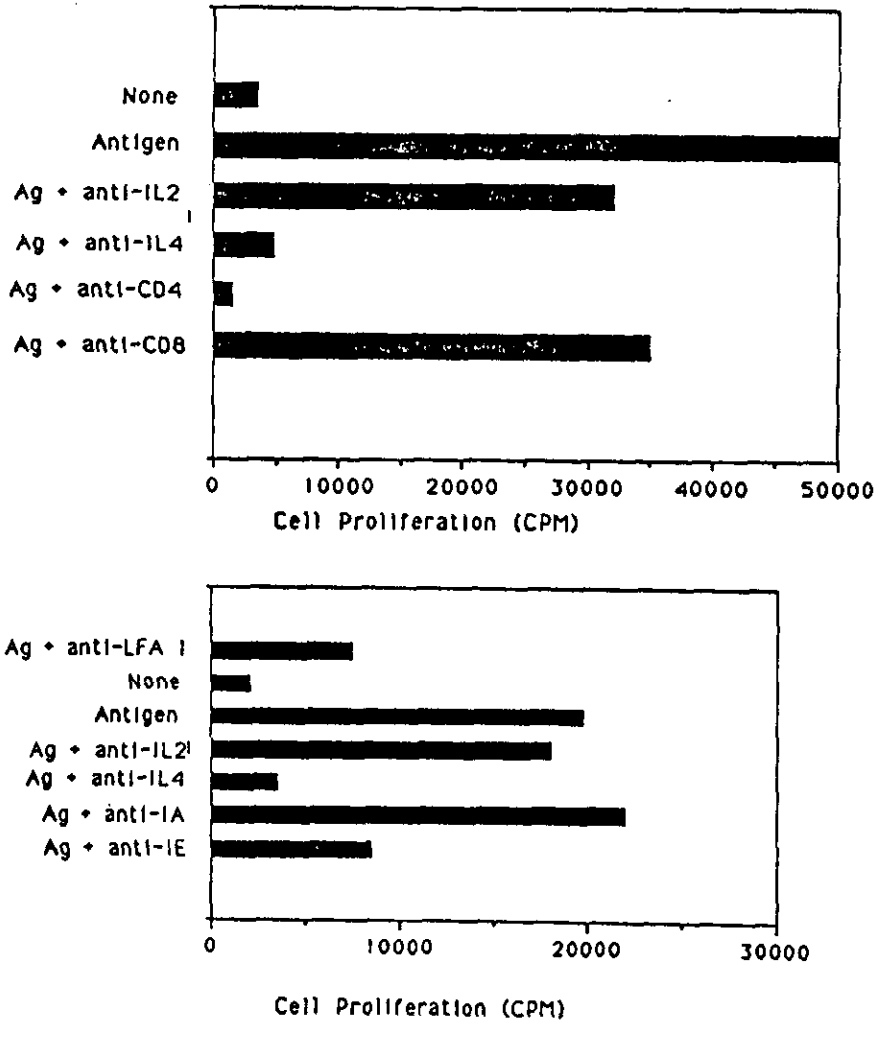


FIGURE 7
A: Treatment of cloned cells with anti interleukin-4 abolishes antigen recognition and cell proliferation, as does treatment with anti-CD4 monoclonal antibody, indicating that cloned cells are CD4-positive cells that are dependent on CD4, MHC encoded recognition, and that they are also dependent on interleukin-4 for growth. B: Additional characterization studies indicating dependence on this cell line of interleukin-4 and of I-E encoded determinants for growth and proliferation.

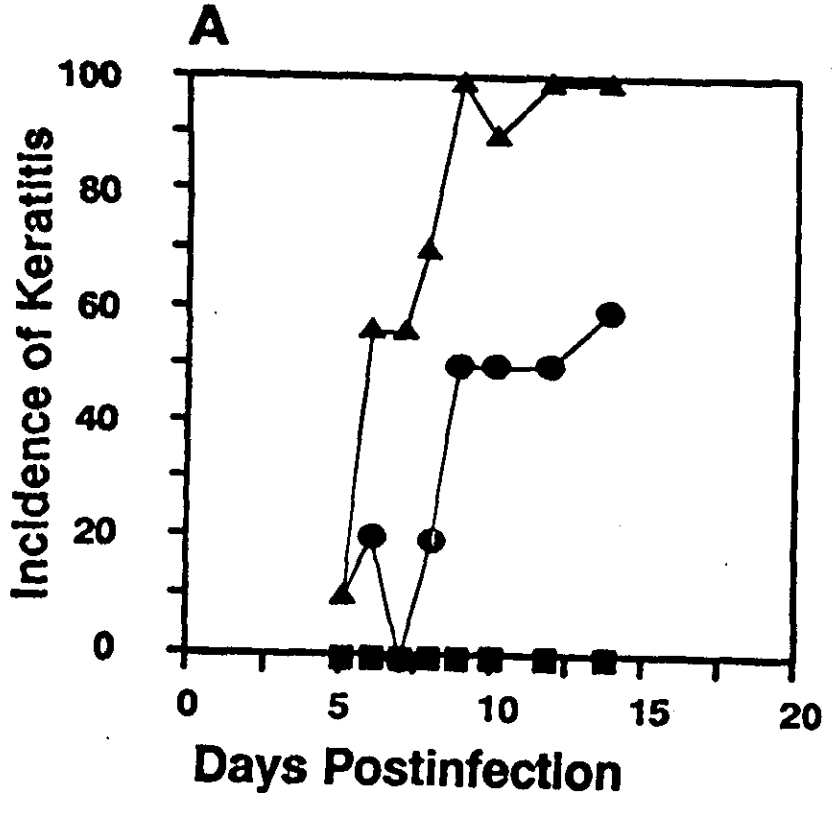


FIGURE 8A
Incidence of keratitis in C.AL-20 mice receiving cloned cells (*triangles*) compared with normal C.AL-20 mice. Note acceleration and worsening, from standpoint of incidence, of keratopathy development, by adoptive transfer of cloned cells.

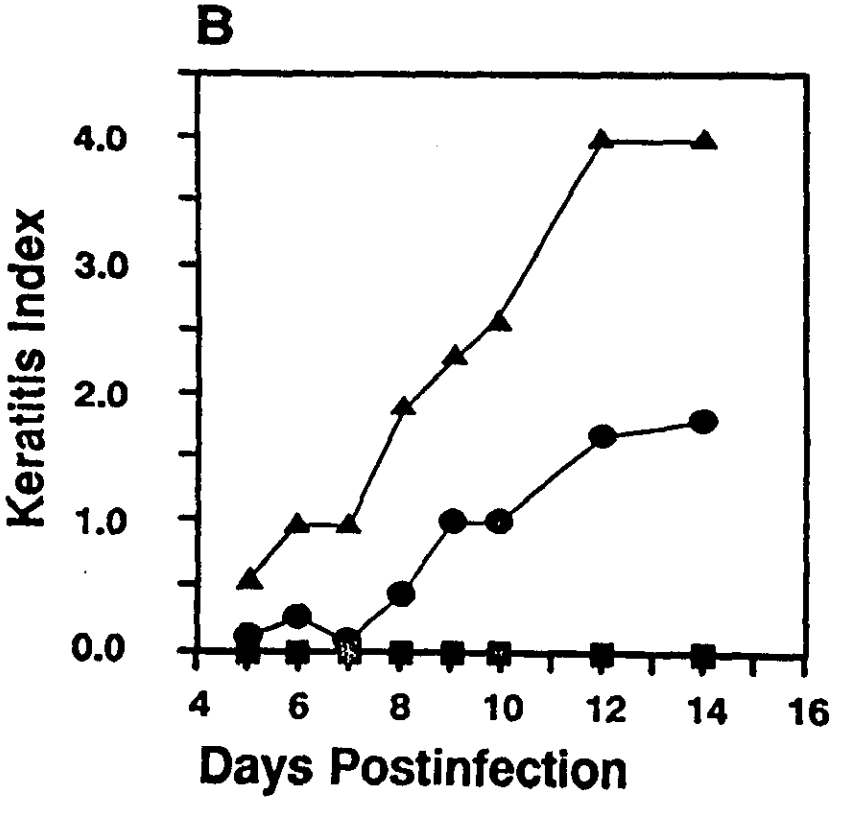


FIGURE 8B
Severity of keratitis in C.AL-20 mice receiving $V_{\beta 8.1,2}$ CD4+ HSV specific T cells. Note rapid development of more severe keratitis in those mice receiving these cells (*triangles*) as compared with those C.AL-20 mice that did not receive these adoptively transferred cells (*circles*).

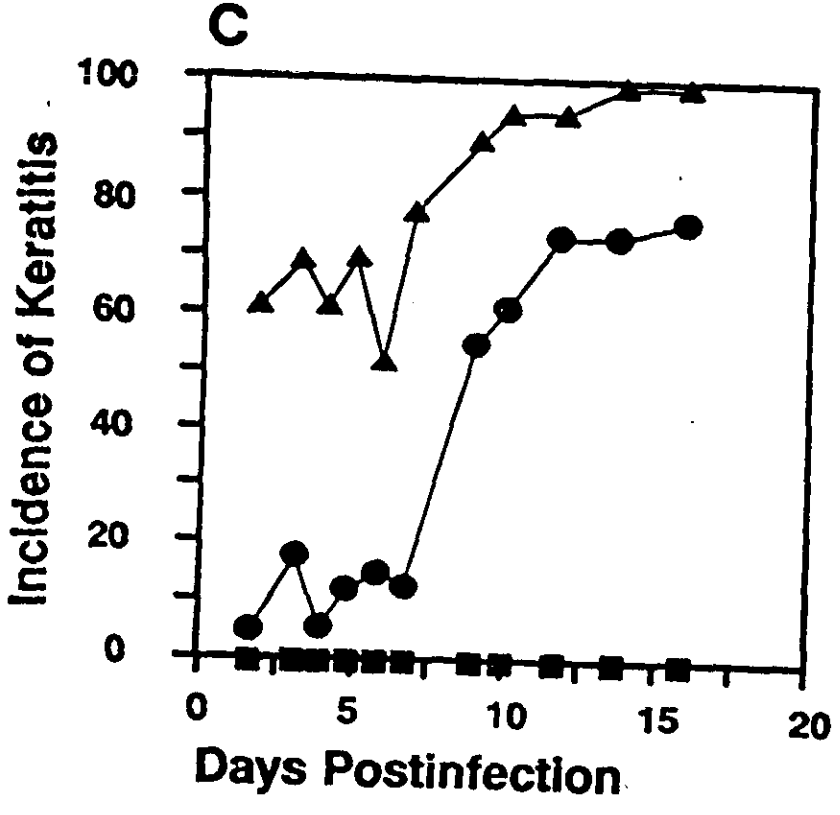


FIGURE 8C
Similar experiments showing increase in incidence of HSK in BALB/c mice receiving transferred cells.

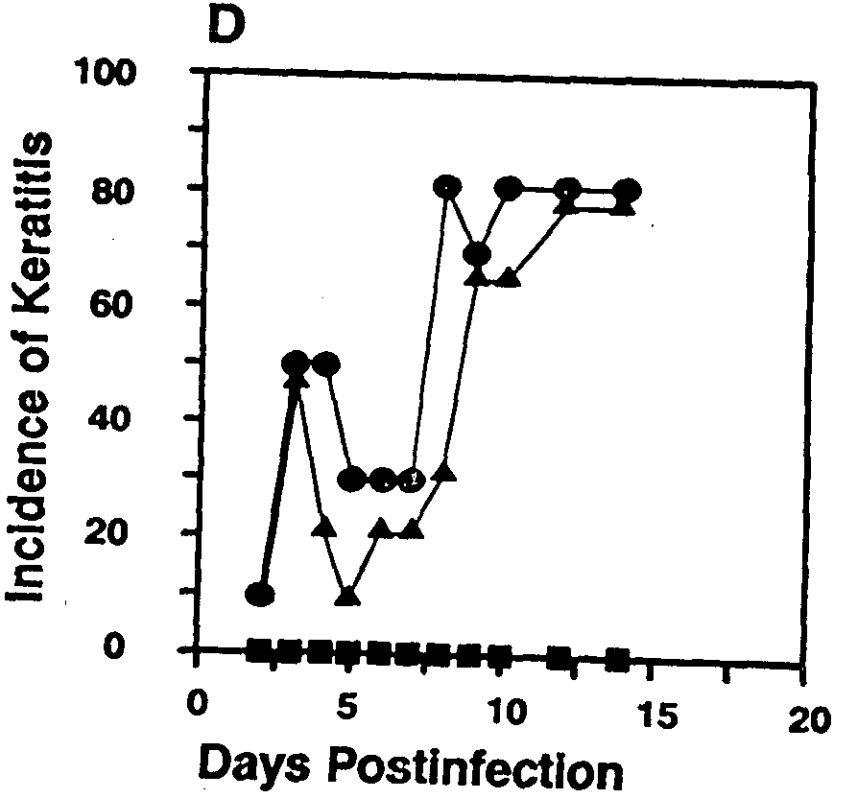


FIGURE 8D
Transfer of PPD-specific T cells into BALB/c mice does not accentuate development of or severity of keratopathy.

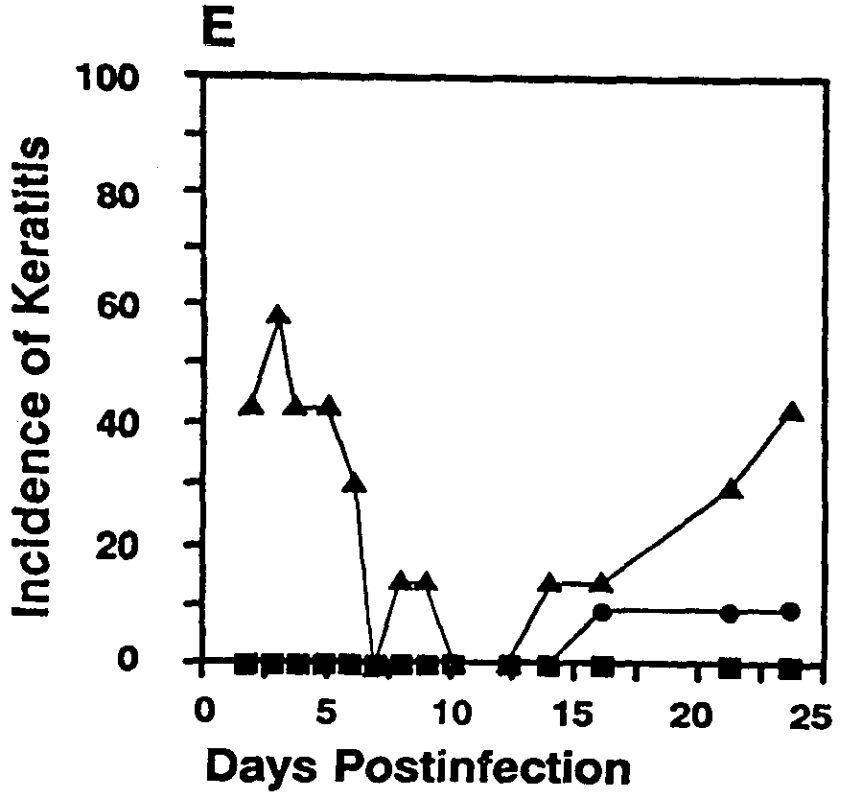


FIGURE 8E
Transfer of cloned herpes-specific T cells into resistant C.B-17 mice transforms them into HSK-susceptible mice.

DISCUSSION

The immune responses in the eyes have developed in ways that are evolutionarily advantageous, ie, allowing immunologic responses capable of eliminating microbes such as herpes from the eye while at the same time preserving the architecture required for continued good vision. This occurs primarily through a highly regulated immunologic response to antigenic material encountered in the various ocular compartments, and this tight regulation of the inflammatory response to antigenic encounter produces characteristics to the immune response that are unique to the eye. It became clear to investigators a decade ago that there existed dramatic differences between animal species and even between various inbred strains of a species in the immune/inflammatory response to antigenic encounter within the eye, including HSV encounter with the cornea.[23] It also became clear, through the pioneering work of Metcalf and Kaufman[2] and others[3-6] that, whereas T-lymphocyte immunity against HSV was or is critical to prevention of death from HSV-induced encephalitis, T cell immunity against HSV is, in large measure, responsible for the destructive keratopathy seen in some strains of mice after corneal inoculation with HSV. Our studies in this area of the immunogenetics of HSK disclosed an extraordinary relationship between susceptibility or resistance to necrotizing HSK and the Igh-1 phenotype. This observation has been confirmed repeatedly by multiple investigators over the past 7 years.

The Igh-1 gene locus on chromosome 12 of the mouse encodes for the synthesis of IgG_{2A} immunoglobulin heavy chain. BALB/c Igh-1 disparate congenic mice (BALB/c-Igh-1^a, C.AL-20-Igh-1^d, and C.B-17-Igh-1^b) are genetically identical except for 20% to 30% of the DNA at and around the Igh-1 locus on chromosome 12. Additional studies in our laboratory over the past 2 years, employing the offspring of multiple backcross breedings, have dramatically narrowed the HSK resistance/susceptibility gene to the Igh-C vicinity. Thus, Igh-C^a genotype is always associated with HSK susceptibility, whereas Igh-C^b genotype is always associated with HSK resistance.[26] In our study of the in vitro immunologic correlates of the Igh-1-linked HSK susceptibility, however, we observed immune response differences not only in the early anti HSV IgG isotype profiles in these Igh-1 disparate congenic mice, but also T lymphocyte behavioral differences. And since it is clear that gene products from the Igh-1 region influence not only antibody responses but also T cell responses, through the presence of T cell-related gene and through idiotype-driven influences on TCR repertoire development, we have also continued to study potential disparate T cell responses in these HSK differentially susceptible Igh-1 disparate congenic mice. Toward that end, we have studied differences in TCR V_β usage (spleen cells and lymph

node cells) and, as described herein, TCR V_β expression by the CD4+ T cells infiltrating the corneas affected by HSK. To our immense surprise, we found, in the HSK-susceptible mice, $V_{\beta 8.1,2}$ TCR almost exclusively on the infiltrating CD4 cells; a small but significant population of $V_{\beta 3}$ T cells was also present. We therefore reasoned that these subsets of T lymphocytes are important contributors to the development of necrotizing HSK. In vivo $V_{\beta 8.1,2}$ and $V_{\beta 3}$ depletion experiments supported this hypothesis, in that depletion of T cells employing $V_{\beta 8.1,2}$ or $V_{\beta 3}$ in the TCR prior to corneal inoculation with HSV abrogated development of necrotizing keratitis. To further investigate the idea that $V_{\beta 8.1,2}$+ CD4+ T cells are responsible, at least in part, for the destructive keratopathy seen in BALB/c (and related) mice after corneal inoculation with HSV, we developed a herpes-specific CD4 T cell clone that employs the $V_{\beta 8.2}$ molecule in its TCR. This clone is responsive to a specific single epitope on one herpes protein, gD. We show, in the experiments described herein, that HSK in C.AL-20 (HSK-susceptible) BALB/c (HSK-intermediate susceptible) and C.B-17 (HSK-resistant) mice is accelerated and worsened by the adoptive transfer of the cells prior to HSV corneal inoculation. Conversely, in experiments reported previously, it was shown that HSV-immune CD8+ T cells are protective against such keratitis.[14]

The clone of T cells we developed after immunizing mice with a gD peptide is a type 2 helper T cell (Th_2 CD4+ T lymphocyte). While Th_1-type helper T cells often appear to play a protective role in infectious disease, Th_2 CD4 T cell responses are often incriminated in the development of excessive host tissue-damaging inflammatory responses in some infectious and allergic conditions.[27,28] Our data demonstrating that a gD-specific Th_2 type T cell is involved in contributing to necrotizing HSK extends the notion of the involvement of Th_2 cells in the production of "innocent bystander" tissue damage. Th_2 cells secrete lymphokines (specifically IL-4 and IL-5) that can attract and activate nonspecific effector inflammatory cells, such as NK cells and macrophages, which can contribute to the tissue damage at the site of inflammation. Indeed, the vast preponderance of infiltrating cells in the corneas affected by HSK in our murine model are CD4 cells, NK cells, and macrophages. This capacity of the CD4+ $V_{\beta 8}$+ Th_2 T cell clone to participate in a disadvantageous inflammatory response in the cornea is highlighted by the fact that after intraperitoneal or intravenous adoptive transfer into normally resistant C.B-17 ($Igh\text{-}1^b$) mice, these cells home to the HSV-inoculated cornea and participate in the development of HSK 2 days after corneal inoculation. The C.B-17 mice that developed HSK after the T cell clone transfer transiently recovered from that HSK but subsequently developed recurrent corneal inflammation, and 40% of the mice

receiving these adoptively transferred cells exhibited significant inflammatory keratopathy; this is in striking contrast to the 7.5% incidence of HSK in normal, untreated C.B-17 mice. It is possible that CD4+ Th_2 cells are not optimally induced in HSK-resistant C.B-17 mice, and perhaps this is why those mice ordinarily enjoy freedom from destructive keratitis after corneal encounter with HSV.

It is clear that the gD epitope to which our T cell clone is specific (gD5-23) can sensitize for elaboration of pathogenic cells, as in the present study, but can also immunize and produce protective immunity.[20] As in any immune response, multiple components of the immune system contribute to the overall symphonic interplay. The fine molecular details that govern the outcome—successful, adaptive or destructive, disease-producing—are the quest of these and other experiments. We believe that it is only through knowledge of these details that effective, selective immunomodulatory therapies can be designed, enabling elimination of virus while at the same time allowing preservation of normal ocular structure and function. Is it possible, for example, that on the basis of the information presented in this report, T cell immunization strategies designed to eliminate $V_{\beta 8.2}$+ CD4+ T cell responses to HSV would convert HSK-susceptible mice into HSK-resistant ones? We suspect that this is so and currently are engaged in work designed to test this hypothesis. We have already shown, for example, that "tolerization" of HSK-susceptible mice with appropriate nonimmune immunoglobulin converts them completely into HSK-resistant ones.[28] Similarly, is it possible that already-established HSK could be ameliorated through anti-$V_{\beta 8.2}$ monoclonal antibody therapy? These and other strategies that may modulate destructive HSK are currently being studied.

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DISCUSSION

DR W. RICHARD GREEN. This study by Dr Stephen Foster and colleagues demonstrates quite conclusively that the presence of T helper cells within the limbal conjunctiva of susceptible mouse strains greatly enhances the clinical inflammatory condition and the pathology of the keratitis after corneal challenge with HSV. These data substantiate earlier observations made by Dr Foster that mice bearing particular Igh-1 genes are more susceptible to HSK than are mice bearing different genes from the same presumed locus. In a series of experiments where T helper cell CD4-positive $V_{\beta 8.1,2}$-positive T cells were depleted, there was a marked reduction in both the incidence and severity of keratitis following corneal inoculation of HSV. In addition, adoptive transfer of CD4-positive $V_{\beta 8.2}$-positive T helper cells responding to a particular HSV peptide greatly accelerated the response of recipient mice subsequently challenged with HSV corneal inoculation. Taken together, these data are sufficient to indicate that the presence of T helper cells specific for HSV protein can hasten the appearance of corneal disease, increase the cell damage caused by the virus, and thus induce a greater degree of immunopathology than could be seen in their absence.

While it is generally accepted that CD4-positive T cells produce inflammatory cytokines following antigen recognition, it should also be borne in mind that CD8-positive T cells are also capable of doing much the same thing, although quantitatively the amount of cytokines produced may be less. The clear distinction between helper T cells and cytolytic T cells is not so sharp when it comes to cytokine production. In the experiments of Dr Foster and co-workers, there was clearly some clinical disease and pathologic damage even in HSV-challenged mice depleted of this T helper cell population.

I would like to congratulate the authors on an excellent study and to ask if they have conducted studies with depletion of both CD4-positive $V_{\beta 8.1,2}$-positive and CD8-positive T cells. I would also like to ask if there is any correlate or potential value of these findings in understanding HSV infections in humans.

ACKNOWLEDGMENT

I would like to acknowledge the help of Judith Whittum-Hudson, PhD, and Robert A Prendergast, MD, in the review of this paper.

DR C. STEPHEN FOSTER. I appreciate Dr Green's kind remarks. In experiments not recorded here we have done selective depletions in susceptible mice and in resistant mice of T cell subset populations. Additionally, we have done adoptive transfer studies with herpes reactive CD4 and CD8 T cells in susceptible and resistant mice. And finally we have done experiments in T cell deficient nude mice in which we have done similar adoptive transfer studies of a selected population of CD4 and CD8 T cells. All of those different strategies trying to get at this issue of which cell type predominantly seems to confer protection and which cell type seems to predominantly participate in the creation of mischief have led us to conclude that, at least in

the mouse system that I reported to you this morning, in general CD4 T cells tend to produce excessive inflammation and CD8 T cells seem to play an immunoregulatory role and control runaway and wild innocent bystanders damaging inflammation. In an experiment also not shown here today I should emphasize that this is a very complicated drama and we don't want to imply that it is primarily the T cell that is the mischief maker. Natural killer cells play a major role in this drama and we have shown that the depletion of natural killer cells from those mice that are quite susceptible to developing keratitis confers total protection against the corneal damage that ordinarily occurs in that model of HSK. We have viewed the CD4 cell and the CD8 cell as primarily regulatory cells, probably through the generation of the particular cytokines that each produces. The question as to how all of this might eventually have some relevance in humans is the whole reason that I got into the business in the first place. The first work that we performed in this research area was in humans and specifically with NK cells; we discovered very quickly that there was little that we could do to try to work out the details of this complicated drama in humans and hence the change to mouse work. It is probably true that humans who exhibit, in the course of your care of them, very great differences in their degree of keratopathy secondary to the herpes, and great differences in their recurrence rates over the course of a decade of observation are telling you that they are having dramatically different immunologic responses to HSV, one patient from the other. We know the chromosome that is comparable to the Igh-containing chromosome 12 in the mouse, is chromosome 14 in humans: we know the general location of the gene that regulates immunoglobulin synthesis, and we intend to study humans after we finish with the mouse work. We fully anticipate that we will find a regulatory gene close to the Igh-1 locus that controls either CD4 cell behavior or NK cell behavior. Is it possible that in those individuals who are exhibiting major destruction secondary to their immunologic response to herpes that vaccination strategies could be of use to them? I don't mean in terms of prevention (it is too late for that, since they are already infected), but rather from the standpoint of regulating T cell responses in those individuals who are having major trouble. Is it possible that this could suppress a particular T cell type that is over-represented in them? We have shown that this is possible in mice. Immunizing with a glycoprotein D peptide results in the dramatic reduction in a proportion of those $V_{\beta 8.1,2}$ CD4 T cells in those susceptible mice. We also think it is possible this may be true in humans and furthermore believe that regulating the cytokines that are being produced by the cells has great promise for the future.

Advances in Ocular Immunology
R.B. Nussenblatt, S.M. Whitcup, R.R. Caspi and I. Gery, eds.

Expression of Vβ T cell receptor (TCR) transcripts in eyes, lymph nodes, and spleen in experimental murine keratitis.

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Introduction

Host immune mechanisms directed toward the elimination of herpes simplex virus (HSV) play a major role in the pathogenesis of HSK. Studies in Igh-1 disparate BALB/c congenic mice, C.AL-20 ($H\text{-}2^d$, $Igh1^d$) HSK-susceptible, and C.B-17 ($H\text{-}2^d$, $Igh1^b$) HSK-resistant mice, have led to the notion that CD4+ T cells are involved in the induction of HSK and CD8+ T cells in the protection from HSK.[1] Most of the CD4+ T cells infiltrating the corneas of susceptible mice were Vβ8.1,2+, and some Vβ3+ were also detected. When C.AL-20 susceptible mice were rendered CD4+ Vβ8.1,2+ or Vβ3+ T cell deficient through in vivo monoclonal antibody therapy, a dramatic reduction was found in both the incidence and the severity of keratopathy following HSV corneal inoculation as compared to controls.[2] In this report we have analyzed the TCR repertoire in both strains of mice using polymerase chain reaction technique.

Material and methods

Corneal inoculation and clinical scoring. C.AL-20 and C.B-17 mice were anesthetized, and the right cornea of each mouse was scratched in a crisscross pattern. Five ul of an HSV-1 (KOS strain) suspension containing $2.5x10^5$ p.f.u. of virus were instilled in the cul-de-sac. The corneas were examined under binocular microscopy for 21 days after inoculation to determine the severity of keratitis.

Oligonucleotide primers and PCR amplification. Sequence specific primers for 19 Vβ families and a primer antisense for the constant region of TCR β chain (Cβ) were chosen from the Gene Bank and synthesized using an automated DNA synthesizer (Pharmacia LKB, Piscataway, NJ).

RNA isolation, cDNA synthesis, and PCR amplification. Inoculated eyes, spleens, and regional lymph nodes were harvested both before, and at various time points after corneal HSV infection (4, 7, 11, 14 and 21 days p.i.). Total RNA was extracted from each organ.[3] cDNA was synthesized and its confirmed using primer specific β-actin, a housekeeping gene that is expressed at a relatively constant level in all cells. PCR was performed by using a wax pellet hot-start technique. The amplification was performed in a Perkin Elmer thermal cycler model 9600 (Perkin Elmer Cetus, Norwalk, CT) with the following profile: 30 cycles of 1' at 94°C, 1' at 55°C, and 2' at 72°C.

Southern blot analysis and scanning densitometry. PCR products were electrophoresed in 1.5% agarose gels, visualized with ethidium bromide and blotted onto nylon membrane (Boehringer). Densitometry was used to determine the differences between Vβ TCR transcripts of the same family members in HSK-susceptible and HSK-resistant mice.

Results

TCR Vβ mRNA expression in spleen. In the spleen more Vβ tanscripts were seen in C.B-17 resistant mice as compared with C.AL-20 mice. The pattern of Vβ transcripts expressed was different from that seen in eyes and LN. (data not shown).

TCR Vβ mRNA expression in LN. At later time points (11, 14 and 21), more Vβ transcripts were found in regional LN of C.AL-20 mice as compared to C.B-17 (data not shown). Only at day 14, densitometer analysis showed differences in the Vβ/Cβ ratios for Vβ8.1, Vβ8.2 and Vβ8.3: greater

intensity in the message was detected for Vβ8.1 and Vβ8.2 in LN of C.AL-20 mice, while C.B-17 mice showed a greater intensity for Vβ8.3. (Fig. 1).

TCR Vβ mRNA expression in eyes. As was seen in LN, at day 11, 14 and 21 more Vβ transcripts were expressed in the inoculated eyes of C.AL-20 mice as compared to C.B-17 mice. Vβ8 family members were seen in both strains of mice at the later time points (Table 1). At day 11, in C.AL-20 mice Vβ8.2 and Vβ8.3 showed a significantly greater intensity in the message, when compared to C.B-17 mice. (Fig. 2). At day 11, Vβ 8.1 was expressed only in C.B-17 mice. At day 14, both strains of mice showed mRNA gene expression of the three family members with not significantly different densitometer ratios (data not shown).

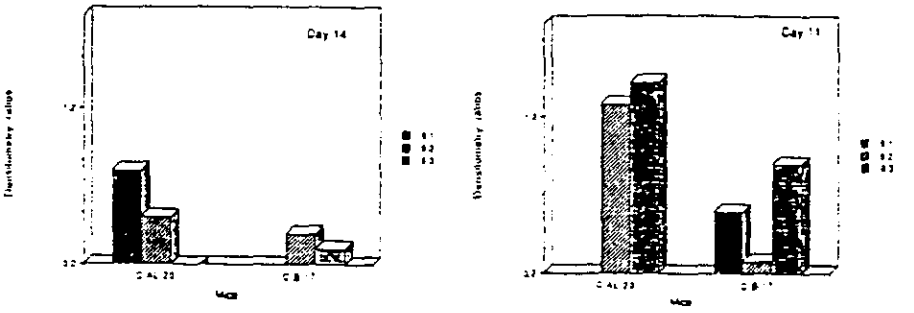

Fig. 1 and 2. Densitometry ratios of Vβ/Cβ in LN (left) at day 14, and inoculated eyes (right) at day 11 after corneal inoculation with HSV.

Table 1. TCR Vβ transcripts in eyes. A) C.AL-20 mice ($Igh\text{-}1^{a}$), and B) C.B-17 mice ($Igh\text{-}1^{b}$).

A	Vβ																						
D	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
0										+								+				
4			+			+	+		+	+							+	+				
7	+	+	+				+			+	+	+	+				+	+	+			
11	+	+		+	+		+	+		+	+	+	+	+	+	+	+	+	+			+
14	+	+		+			+	+	+	+	+	+	+			+	+	+	+			+
21	+	+		+	+		+	+	+	+	+	+	+	+	+	+	+	+				+
B																						
0			+							+	+						+	+				+
4	+		+						+	+	+						+					
7	+	+		+			+		+	+	+						+	+	+			+
11	+								+	+	+						+					
14									+	+	+			+				+				+
21						+	+	+		+	+		+			+		+				+

Transcription of Vβ genes in congenic mice at several time points as detected by PCR and Southern blot. "+" signifies Vβ mRNA detected, while blank means absence. D=day.

Discusion

Attempts to define the participation of different Vβ T cell receptor families have been made in many experimental diseases.[4] The interpretation of the data obtained has not been conclusive. We found that BALB/c congenic Igh-1 disparate mice, differentially HSK susceptible have different TCR usage kinetics in response to HSV corneal inoculation. In general, more diverse Vβ mRNA expression was detected in eyes and LN from HSK-suscpetible mice as compared to HSK-resistant mice. Densitometry readings showed evident differences in the intensity of the message in Vβ8 family members at several, later time points. Our findings at the level of gene expression correlate with our previous results regarding the probable participation of Vβ8+ T cells in the pathogenesis of HSK.

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T CELL RECEPTOR Vβ GENE EXPRESSION IN EXPERIMENTAL HERPES STROMAL KERATITIS

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SUMMARY

Our study examined T cell receptor (TCR) Vβ mRNA expression in a murine model of experimental herpes simplex keratitis (HSK). We employed a polymerase chain reaction (PCR) technique to detect TCR Vβ 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 Vβ transcript usage pattern was detected as compared with that seen in HSK-resistant C.B-17 mice. Vβ8 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 Vβ8.2 and Vβ8.3 message was significantly greater in the eyes of C.AL-20 mice; Vβ8.1 was expressed only in C.B-17 mice. There were obvious differences in the TCR Vβ expression between HSK-susceptible and HSK-resistant mice. The differences in the intensity of the message expressed by Vβ8 family members between the two strains could be correlated to previous experiments that showed Vβ8.1,2+ 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, we[1] and others[2-5] 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 ($H\text{-}2^d$, $Igh\text{-}1^d$) mice develop severe keratopathy, whereas C.B-17 ($H\text{-}2^d$, $Igh\text{-}1^b$) 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) Vβ usage in the keratitis model showed a restricted repertoire of T cells infiltrating the corneas of susceptible mice; immunohistochemical studies showed that Vβ8.1,2+ T cells were the main infiltrating cells by day 11 and 14 after challenge with HSV-1, with fewer numbers of Vβ3- and Vβ6+ cells seen.[9] Adoptive transfer of a herpes glycoprotein-D peptide specific, CD4+ Vβ8.2+, 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 Vβ8+ or Vβ3+ 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 Vβ 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 Vβ 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 Vβ 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

Vβ	Sequence	PCR product (bp)
Vβ1	TGATTCGAAATGAGACGGTG	458
Vβ2	GTGCTGATTACCTGGCCACA	440
Vβ3	GAGTGGAGTCAGAAGTCGAT	450
Vβ4	CGCTTCCAACCTCAAAGTTC	442
Vβ5.1	GAGAGATAAAGGAAACCTGC	310
Vβ5.2	AGCAATGTGGTCTGGTACCAGA	580
Vβ6	ATGATGCGTCTCGAGAGAAG	436
Vβ7	AGATGGTGGGGGTTTCAAGG	448
Vβ8.1	GTCCAAGAAGCAAGGTGGCA	613
Vβ8.2	TGCTGGCAGCACTGAGAAA	428
Vβ8.3	TAGAAACAAGGTGACAGTAACA	614
Vβ9	TTCCAATCCAGTCGGCCTAA	436
Vβ10	TCTCACCTCAGTCTTCAGAT	439
Vβ11	AACGATTCTCAGCTCAGATG	434
Vβ12	CGATTCAAAGCTGAGATGCT	432
Vβ13	TCTGCTGTGAGGCCTAAAGG	436
Vβ14	CTGAACCTCTCAGCTTCCAG	439
Vβ15	CCATCAGTCATCCCAACTTA	432
Vβ16	CAGTTGAAAGACCAGATGGT	427
Vβ17	GATTCTCAGCTAAGTGTTCC	432
Vβ18	CAATCAGCCGGCCAAACCTA	436
Vβ19	TGTACTTCTGTGCTAGCAGTC	290
	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 Vβ 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 μg (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 μl 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 Vβ families and a primer antisense for the constant region of TCR β chain (Cβ) 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 Biotecx 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 μl. 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 Vβ 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 Vβ transcripts in eyes of C.AL-20 mice (Igh-1^a) and C.B-17 mice (Igh-1^b)

	Vβ																					
Day	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
C.AL-20 mice																						
0										+								+				
4			+			+	-		+	+							+	+				
7	+	+	+				-			+	+	+	+				+	+	+			
11	+	+		+	+		+	+		+	+	+	+	+	-	+	+	+	+			+
14	+	+		+			-	+	+	+	+	+	+			+	+	+	+			+
21	+	+		+	+		-	+	+	+	+	+	+	+	+	+	+	+				+
C.B-17 mice																						
0			+							+	+						+	+				+
4	+		+						+	+	+						+					
7	+	+		+			-		+	+	+						+	+	+			+
11	+								-	-	+						-					
14									-	-	+			+				-				-
21						+	-	-		-	+		-			+		+				+

Expression of Vβ TCR in congenic mice at several time points detected by PCR. '+' indicate presence of Vβ!, 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 $MgCl_2$, 200 μM 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 μl of cDNA. The total reaction volume was 50 μl 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 Cβ probe internal to all the PCR amplified sequences, then washed with 2× SSC/0.01% SDS followed by a final high-stringency wash with 0.5× 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 Vβ TCR transcripts of the same family members in HSK-susceptible and HSK-resistant mice. The relative intensities of the Southern blot band from each Vβ amplification were measured in an ImageQuant densitometer (Molecular Dynamics, Sunnydale, CA) and were compared with the Cβ values for the same sample. The results are expressed as the ratio of the densitometry value for the particular Vβ TCR to the densitometry value for the Cβ TCR.

RESULTS

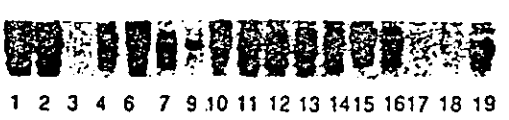
Stromal keratitis developed in only 7.4% of C.B-17 mice by day 11 and persisted through day 21 post-inoculation, while it was present in 85.2% of C.AL-20 mice by the same day post-infection.

More diverse Vβ 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 Vβ transcripts found at 11 and 14 days post-inoculation. At day 11 in C.AL-20 mice, Vβ2, Vβ4, Vβ5.1, Vβ6, Vβ9 to Vβ13, Vβ15, Vβ16 and Vβ19 were expressed (Fig. 1); in C.B-17 mice, those Vβ transcripts were not detected (Table II). At day 14, Vβ1, Vβ2, Vβ4, Vβ6, Vβ7, Vβ9, Vβ10, Vβ13, Vβ14 and Vβ16 were expressed in C.AL-20 mice (Fig. 2) but were absent in C.B-17 mice (Table II). At day 21 post-inoculation, Vβ1, Vβ2, Vβ4, Vβ5.1, Vβ6, Vβ7, Vβ8.1, Vβ9, Vβ11, Vβ12 and Vβ14 were transcribed in C.AL-20 mice but not in C.B-17 mice (Table II).

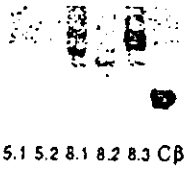
The expression of Vβ8 family members was seen in both strains of mice at days 11, 14 and 21. However, the intensity of expression of Vβ8.2 and Vβ8.3 was different between these strains of mice at day 11, the time of onset of keratitis. The densitometry readings showed a significantly greater intensity in Vβ8.2 and Vβ8.3 message in C.AL-20 mice compared with C.B-17 mice (Fig. 3). Vβ8.1 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 Vβ8.1, Vβ8.2 and Vβ8.3 (Fig. 2), with different but not significantly different densitometer ratios (Fig. 4). The proportion of Vβ8.2 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

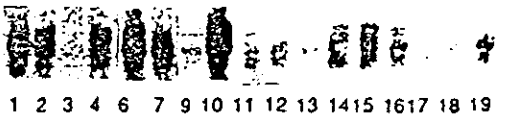


C.AL-20



C.B-17

Fig. 1. *TCR Vβ 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. Vβ1 to Vβ19 from C.AL-20 mice are shown, as well as Vβ5 and Vβ8 family members for each mouse strain.*



C.AL-20

C.B-17

Fig. 2. *TCR Vβ 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. Vβ1 to Vβ19 from C.AL-20 mice are shown, as well as Vβ5 and Vβ8 family members for each mouse strain.*

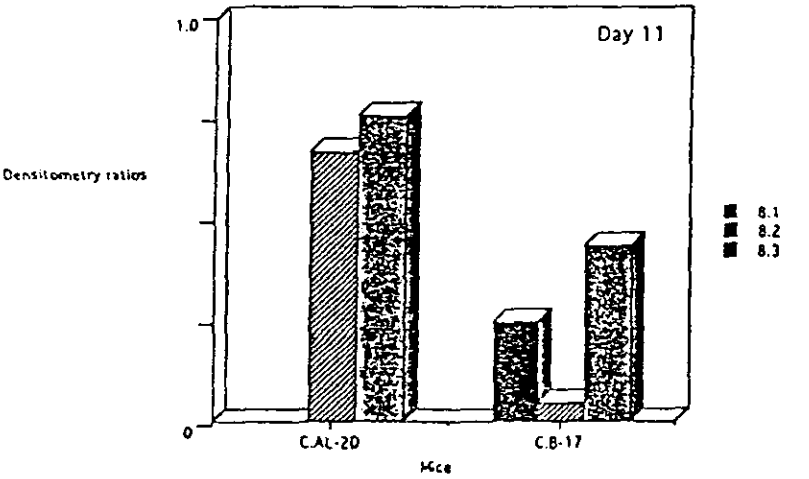


Fig. 3. *Autoradiograms were quantified by image analysis as described in Methods. Vβ/Cβ ratios of Vβ8 family members, obtained from eyes at day 11, are shown.*

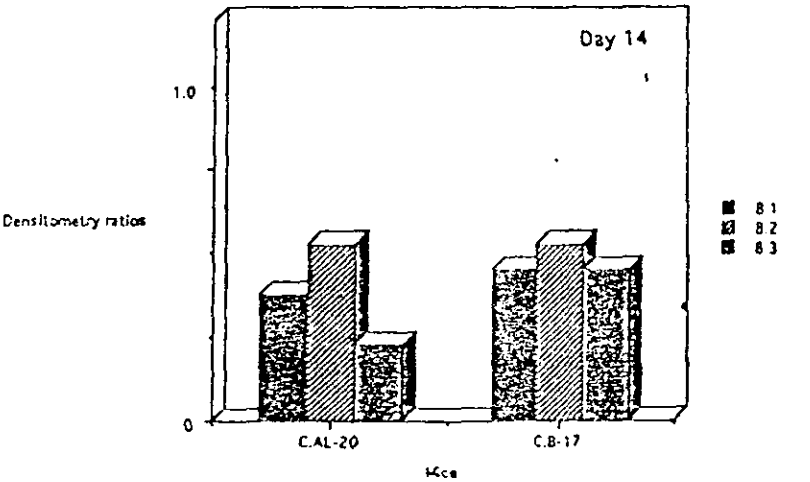


Fig. 4. *Autoradiograms were quantified by image analysis as described in Methods. Vβ/Cβ ratios of Vβ8 family members, obtained from eyes at day 14, are shown.*

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 mice[7] or nude mice[8] 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 Vβ usage has been shown. Studies have consistently shown that specific monoclonal antibodies and vaccination with specific T cells directed against Vβ8 TCR protects animals from demyelinating disease (for review see Vandenbark *et al.*[26]). We previously reported that T cells bearing Vβ8.1,2 TCR, and to a lesser extent Vβ3 and Vβ6, 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 Vβ transcripts were observed in eyes from HSK-susceptible mice, when compared with HSK-resistant mice, after HSV-1 corneal inoculation. Vβ5.1, Vβ5.2, Vβ7, Vβ8.2, Vβ8.3, Vβ9 and Vβ12 were seen in eyes from C.AL-20 mice, but not at any time point in C.B-17 mice.

The diverse Vβ transcripts found in the eye did not represent a specific Vβ 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 Vβ 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 Vβ transcripts were seen in eyes from HSK-susceptible mice.

Our data clearly show a diverse TCR Vβ 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 Vβ8 family members between the Igh-1 disparate congenic strains of mice; increased intensity in the message in Vβ8.2 and Vβ8.3 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+ Vβ8.2+, IL-4 producing Th2 clone,[10] and the findings that specific Vβ8 T cell subset depletion by using therapy with monoclonal antibodies prevents HSK development in HSK-susceptible mice.[1] The differences that we found in Vβ8 usage at the level of mRNA expression further support a role for the Vβ8 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 Vβ 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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DISCUSIÓN

Existen diferentes entidades infecciosas donde la respuesta inmune celular dsempeña un papel en la inmunopatogénesis, como es el caso de la inmunopatología del ojo (39-45). La respuesta inmune en el ojo, desencadenada en contra de algun antígeno determinado, requiere de una intrincada inmunoregulación, ya que debe eliminar al antígeno desencadenante y, al mismo tiempo, preservar la arquitectura y función oculares. En lo particular, hemos estudiado una patología con compromiso de la respuesta inmune celular a nivel ocular inducida por los mecanismos de defensa al hongo intracelular *Histoplasma capsulatum.* Antecedentes experimentales en animales y evidencias en humanos demuestran daño ocasionado por la respuesta inmune en una lesión tardia que ocasiona un síndrome ocular denominado síndrome de histoplasmosis ocular presuntiva (SHOP) (41).

En el modelo viral, Metcalf y cols (25) y otros (9, 15, 26-29) demostraron que la inmunidad mediada por linfocitos T contra del VHS es crítica para prevenir la muerte por encefalitis experimental, sin embargo, es la responsable de la queratopatía destructiva observada en algunas cepas de ratones después de que el virus se inoculó en la córnea; estos datos evidencian que la respuesta inmune bajo diferentes estímulos y mecanismos puede ejercer efectos polares, uno protector y otro dañino.

En el presente trabajo se corroboró que los linfocitos T CD4+ son el principal tipo celular involucrado en la queratitis mediada por el VHS, en ratones

congénicos BALB/c (BALB/c-Igh1^{a}, C.AL-20-Igh-1^{d}, y C.B-17-Igh-1^{b}), los cuales son genéticamente idénticos, excepto por su diversidad (20-30%) en el locus Igh-1 (Articulo 1, ref. 47: Fig. 1 y tabla II, pag. 333). El genotipo Igh-Ca está siempre asociado con susceptibilidad a la QHE, mientras que el genotipo Igh-Cb se asocia con resistencia a la QHE (46). Es claro que los productos genéticos de la región Igh-1 no sólo influyen en la respuesta de anticuerpos, sino también, en la respuesta de células T sobre el desarrrollo del repertorio del TCR, razón por lo cual, estudiamos las respuestas potenciales entre las diferentes células T en los ratones con distintas susceptibilidades a la QHE.

Los resultados del presente trabajo demostraron que los ratones susceptibles a la QHE presentaron infiltrados celulares principalmente de linfocitos T CD4+ Vβ8.1,2+ y también de linfocitos T CD4+ Vβ3+ (Articulo 1, ref. 47. Fig. 2 pag. 334). La eliminación de linfocitos T con anticuerpos monoclonales contra los linfocitos T Vβ8.1,2 y Vβ3, influyó en el desarrollo de la QHE en los ratones que tienen diferentes grado de susceptibilidad (Articulo 1, ref. 47. Fig. 4, 5 y 6 pags. 336, 337 y 338). Para apoyar el hallazgo de que los linfocitos T CD4+ Vβ8.1,2+ son responsables de la queratopatía por VHS, se desarrolló una clona de células T específica para un epítope de la glicoproteína D (gD 5-23) del VHS, la cual exacerbó la severidad de la queratitis en los ratones susceptibles C.AL-20 y favoreció el desarrollo de la queratitis en los ratones resistentes C.B-17 (Articulo 1, ref. 47, Figs: 8A-8E, pags. 340-344).

Los estudios que apoyan los diferentes repertorios del TCR que participan en la manifestación de otras enfermedades, en humanos y en modelos experimentales, han sido contradictorios. Algunos autores muestran evidencias de una respuesta restringida de células T (48-55), mientras que otros reportan una diversidad en el rango de la expresión genética del TCR (56-59). Por ejemplo, en la encefalomielitis autoinmune experimental, se ha demostrado una restricción para Vβ; los estudios de Vandenbark AA y cols, sugirieron que el tratamiento con anticuerpos monoclonales y la transferencia con linfocitos T específicos para TCR Vβ8, protegieron a los animales de la enfermedad desmielinizante (60).

Debido a nuestros hallazgos de que los linfocitos T Vβ8.1,2 y Vβ3 son las principales células en los infiltrados de las córneas de los ratones susceptibles a VHS, consideramos importante analizar los diferentes transcriptos de Vβ, mediante PCR a partir de muestras del ojo de los ratones susceptibles y resistentes a la QHE. En general, observamos una mayor diversidad en los Vβ de los linfocitos infiltrados en las córneas de los ratones susceptibles, que en los resistentes. Los diferentes transcriptos de Vβ no representan una subfamilia Vβ específica que podria ser responsable de la inmunopatogénesis de la QHE (Articulo 3, ref. 61, Tabla II y Figs. 1-4, pags. 601 y 602). Estos resultados demostraron una expresión genética diversa de Vβ de TCR, lo que concuerda con resultados de otros autores, quienes no encontraron un repertorio restringido de TCR, usando diferentes modelos experimentales (56-59).

La detección de los mensajeros de Vβ8.2 y Vβ8.3 en los linfocitos procedentes de los ojos de los ratones susceptibles C.AL-20, comparados con los de los ratones resistentes C.B-17, fue más elevado al día 11 de la infección con el VHS. Esto es interesante, según nuestros resultados previos, que demostraron una influencia notoria de la clona de linfocitos Th2 CD4+Vβ8.2+ sobre la patogénesis de QHE (47, 61).

El presente trabajo apoya evidencias de la participación de la inmunidad celular en el daño corneal en ratones infectados por el VHS. Sin embargo, deben analizarse la participación de citocinas liberadas en el microambiente de la QHE que posiblemente desempeñan un papel crítico en la inmunoregulación que ocurre en el daño corneal en los ratones infectados.

CONCLUSIONES

1.- El estudio inmunohistoquímico de las córneas de los ratones susceptibles a la QHE, demostró infiltrado celular, principalmente de linfocitos T CD4+ Vβ8.1,2, y linfocitos T Vβ3, y sugiere que estas células desempeñan un papel importante en el desarrollo de la QHE.

2.- La eliminación *in vivo* de los linfocitos T Vβ8.1,2 ó Vβ3 con anticuerpos monoclonales, antes y después de la inoculación corneal del VHS, evitó el desarrollo de QHE en ratones susceptibles.

3.- Los estudios de transferencia adoptiva de linfocitos T que reconocen un epítope de la glicoproteína gD del VHS, demostraron que la clona de linfocitos T CD4+, Vβ8.2+ aceleró el inicio y acentuó la severidad de la queratopatía en los ratones susceptibles.

4.- Los resultados de PCR demostraron una amplia diversidad en los transcriptos de Vβ en los linfocitos del infiltrado de las córneas de los ratones con QHE. Sin embargo, al día 11 post-inoculación corneal del VHS-1, encontramos diferencias en los miembros de la subfamilia Vβ8 entre los ratones susceptibles y los resistentes, ya que se detectó un aumento en los transcriptos para Vβ8.2 y Vβ8.3 en los ratones susceptibles C.AL-20, pero no en los ratones resistentes C.B-17.

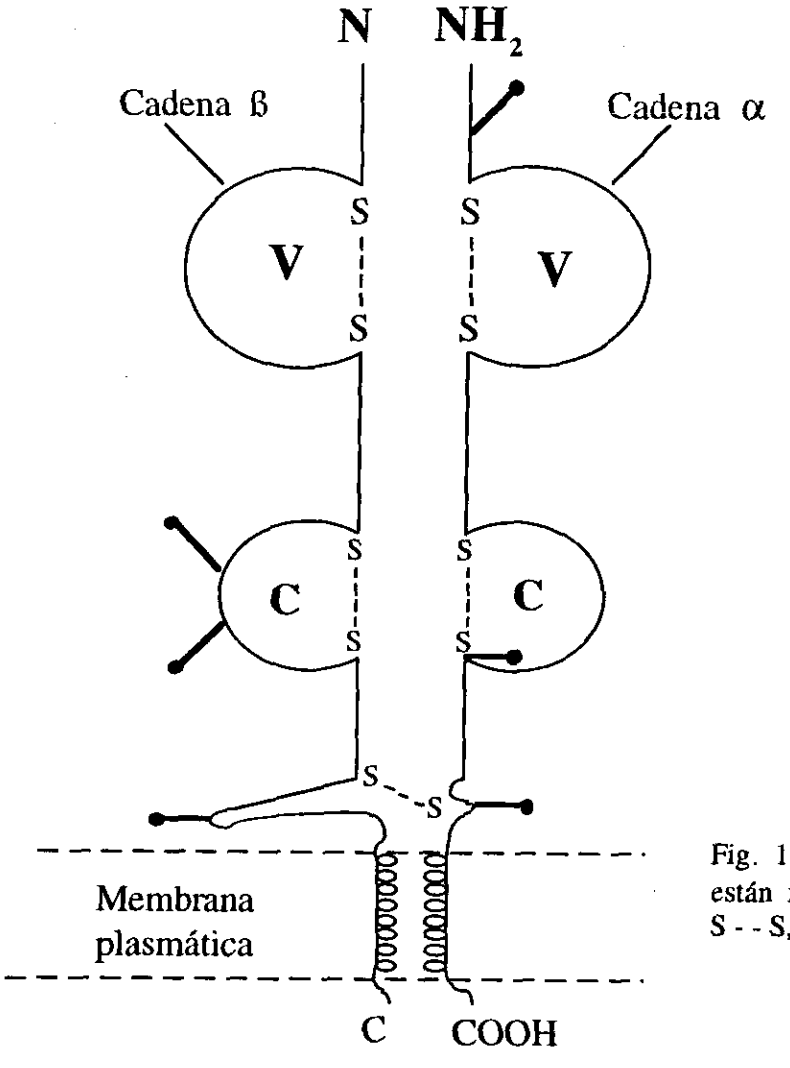

Fig. 1. Diagrama del receptor del linfocito T (TCR). Las cadenas α y ß están formadas por los dominios constantes (C) y variables (V).
S - - S, uniones disulfuro y ——● , localización de carbohidratos.

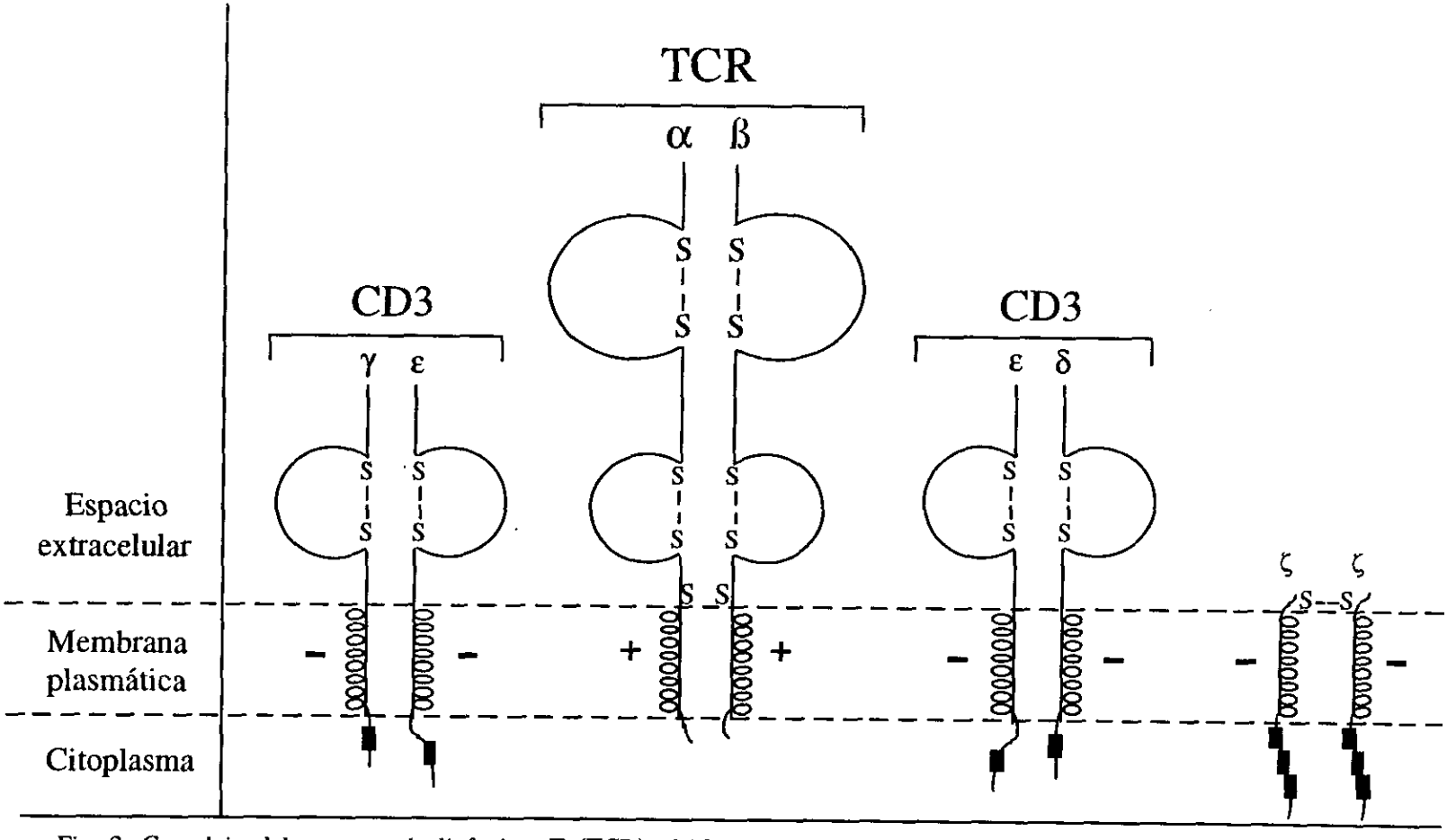

Fig. 2. Complejo del receptor de linfocitos T (TCR). CD3 incluye a las cadenas γ, δ y ε, presentes como monómeros unidos por enlaces no covalentes al heterodímero α β. La cadena ζ está presente como homodímero unido por puentes disulfuro. Los signos + y - se refieren a residuos de aminoácidos con carga en la región transmembranal. ▮ : inmunoreceptor de activación (ITAMs) en las porciones citoplásmicas del CD3 y ζ.

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