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Immunogenetic Influence of Igh-1 Phenotype on Experimental Herpes Simplex Virus Type-1 Corneal Infection

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Patterns of herpes simplex virus type-1 (HSV-1) infection were studied in BALB/c congenic, Igh-1 disparate murine strains to establish the influence of Igh-1 phenotype on the development of keratopathy, trigeminal ganglionic latency and keratocyte permissivity. Eighty-two percent of C.AL-20 (Igh-1^d) mice, 40% of BALB/cByJ (Igh-1^a) mice and 12% of the C.B-17 (Igh-1^b) mice developed herpes simplex keratitis (HSK) following corneal challenge with 2.5×10^4 PFU HSV-1 strain KOS. While disease frequency was directly proportional to HSV-1 challenge dose, relative resistance and susceptibility patterns in the congenic mice were constant and highly significant. F1 progeny from C.AL-20 × C.B-17 matings demonstrated the HSK pattern of the C.B-17 parent suggesting that Igh-1 linked resistance to HSK is dominantly inherited. Equivalent trigeminal ganglionic latency was established following ocular HSV-1 inoculation in the three congenic Igh-1 disparate murine strains. Cultured keratocytes from the three Igh-1 disparate murine strains demonstrated equivalent in vitro permissivity to HSV-1 replication. These data illustrate a strong correlation between Igh-1 phenotype and the development of a HSK in congenic mice. The susceptibility/resistance to HSK in these mice is unrelated to trigeminal ganglionic latency or keratocyte permissivity. Invest Ophthalmol Vis Sci 29:749–754, 1988

Host genetics have a direct influence on both innate and acquired resistance to the development of virus-induced pathology.[1–4] This protection may be related to augmented clearance of virus or through modulation of the immune response to specific viral antigens. Gene products encoded by loci other than the major histocompatibility complex (MHC) have been shown to be responsible for strain-specific resistance patterns to HSV infection in certain inbred mouse strains.[2,4,5] Although in vitro viral replication studies indicate that this resistance is recessively inherited and may involve fibroblast or keratocyte permissivity, in vivo data support a dominant inheritance and an immune mechanism.[4–7] We have previously illustrated the influence of the Igh-1 locus on chromosome 12 in the mouse on HSK, delayed type hypersensitivity and lymphocyte proliferation following corneal HSV-1 challenge.[4,7,8] Inbred mouse strains with Igh-1^d or Igh-1^c consistently develop a severe necrotizing stromal keratitis while animals with the Igh-1^b locus demonstrate a relative resistance to HSK following unilateral corneal challenge. Animals with the Igh-1^a phenotype appear to have an intermediate response. Disease modulation from unrelated gene products cannot be excluded by the inbred murine strains employed in these studies. More importantly, a BALB/c congenic mouse with a resistant phenotype from a C57BL/6J mouse (Igh-1^b) was not available for the previous investigation. In this communication, we report clinical disease patterns, ganglionic latency and keratocyte permissivity in three BALB/c congenic mice with susceptible (Igh-1^d), intermediate (Igh-1^a) and resistant (Igh-1^b) Igh-1 phenotypes from inbred murine ancestry.

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Materials and Methods

Virus

HSV-1 KOS strain was obtained from Dr. David Knipe (Harvard Medical School, Boston, MA) and passed twice in Vero cells (CCL 81 American Type

Cell Collection, Rockville, MD). Infected Vero cell monolayers were harvested when a 4+ cytopathic effect was observed. The infected cells were freeze-thawed three times and centrifuged at 1500 *g*.[7] Supernates were aliquoted and stored frozen at −70°C. Aliquots were selected at random and assayed using a standard plaque assay technique on Vero cells as described previously.[7] Titers were verified in triplicate.

Vero Cell Culture

Vero cell monolayers were maintained in 75 cm^2 flasks (Falcon Plastics, Fisher Scientific Inc., Pittsburgh, PA) using Minimum Essential Medium (MEM) with Earle's Salts containing 10% fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, NY), 0.58 mg/ml L-glutamine (GIBCO), 25 μg/ml Fungizone (Flow Laboratories, McLean, VA), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO). Confluent Vero cell monolayers were trypsinized and plated onto six-well, 9.62 cm^2 tissue culture plates (Linbro, Flow Laboratories, McLean, VA) for viral plaque assays.

Animals

BALB/cByJ (Igh-1^a) mice were obtained from Jackson Laboratories (Bar Harbor, ME). C.AL-20 (Igh-1^d) mice were obtained from Dr. Alfred Nisonoff (Brandeis University, Waltham, MA) and bred in microisolators mounted in a ventilated animal rack in our animal facility. C.B-17 (Igh-1^b) mice were kindly provided by Dr. Charles Sidman (Jackson Laboratories, Bar Harbor, ME).

In certain experiments F1 progeny from C.AL-20 female (Igh-1^d) × C.B-17 male (Igh-1^b) crosses were employed. Eight- to 10-week-old sex-matched mice were used in all experiments. Animals were handled in accordance with the ARVO Resolution on the Use of Animals in Research and NIH guidelines.

Inoculation

Animals were anesthetized with ether and, under binocular microscopy, the right eye of each mouse was scratched with a 23-gauge needle (eight horizontal and eight vertical scratches). Five microliters of an appropriately diluted suspension of HSV-1 (strain KOS) were deposited onto the scarified cornea to effect viral challenges of 1.25×10^4, 2.5×10^4 and 5.0×10^4 PFU per eye. Pilot studies employing different viral challenge doses were necessary to determine the optimal range for HSK frequency in BALB/c mice. The above three doses produced low, intermediate and high frequencies of HSK in Balb/c mice and were used to compare the congenic animals.

Clinical Scoring

Inoculated mice were examined clinically using a binocular operating microscope and were scored in a masked fashion at days 2, 4, 7, 9, 11, 14 and 21. The geographic area of the involved eyelids was assessed and scored from 0 to 4+. A clinical score of 1+ was consistent with less than 25%, 2+ with less than 50%, 3+ with less than 75% and 4+ with 75–100% of the eyelid margin infected. Stromal keratitis was also graded from 0 to 4+ with a score of 1+ consistent with less than 25%, 2+ less than 50%, 3+ less than 75% and 4+ between 75–100% corneal opacity with corneal neovascularization and thinning. Epithelial disease was evaluated and scored 0–4+ using a similar grading system. Animals with representative disease were photographed under the operating microscope and the clinical results tabulated.

Cocultivation-Ganglionic Latency

Trigeminal ganglia ipsilateral to the infected cornea were removed aseptically from mice 21 days following HSV-1 inoculation. Individual ganglia were placed in coculture with confluent Vero cell monolayers and maintained with fresh media changes every other day. The cocultures were inspected daily by inverted phase microscopy for latent viral infection as detected by typical cytopathologic changes in the Vero cell monolayer. Cocultures were maintained for 3 weeks.

Keratocyte Tissue Culture

Mice from each inbred and congenic strain were killed by ether and corneas were removed from enucleated eyes with Vannas scissors under an operating microscope. Epithelial and endothelial cell layers were removed aseptically using sterile cotton tip applicators and a #11 blade scalpel. The corneas were irrigated with phosphate-buffered saline (PBS) and five radial corneal incisions were produced using a #11 blade scalpel.[9] Individual corneas were rinsed with culture media consisting of Dulbecco's Modified Eagle Medium with 25 mM HEPES buffer (GIBCO), 15% FBS, 25 μg/ml Fungizone, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO), and placed in 24-well tissue culture plates (Linbro, Flow Laboratories) under sterile stainless steel mesh. Corneal preparations were then incubated in a 5% CO_2 incubator at 37°C with fresh culture media changes made three times weekly. The cell lines were serially passed following treatment with trypsin-EDTA (GIBCO) after the primary keratocyte monolayers were established.

Keratocyte Permissivity Assay

In vitro keratocyte replication kinetics were determined for each established murine cell line (unpub-

lished data). Cultured keratocyte lines of equal passage and with similar doubling times were matched in all experiments. Trypsinized keratocytes (1×10^5 cells per well) were plated on 24-well tissue culture plates (Linbro, Flow Laboratories) with 1.0 ml culture media for 24 hr in order to allow cell attachment. Three monolayers from each murine strain were designated as enumeration controls in each experiment. Prior to in vitro HSV-1 infection, the control monolayers were trypsinized and counted for keratocyte number and viability. Experimental keratocyte monolayers from each murine strain were then rinsed with MEM without FBS for 10 min and infected with HSV-1 in adsorption media consisting of MEM with 5% FBS and antibiotics for 1 hr at a multiplicity of infection (MOI) of five or seven PFU per cell. Following viral adsorption at 37°C, the monolayers were treated with MEM supplemented with 0.1% Gammar (Armour Pharmaceutical Co., Kankakee, IL) for 15 min, washed with MEM without FBS and incubated with 1.0 ml of culture media for various time intervals at 37°C in a 5% CO_2 incubator. The monolayers were harvested using sterile rubber policemen, pipetted into cryotubes (GIBCO) and freeze-thawed three times using liquid nitrogen and a 37°C waterbath. Each sample was carefully thawed to just beyond the ice-ball stage (2–4°C) in each cycle to prevent nonspecific viral inactivation. Individual monolayer homogenates were then centrifuged at 1500 *g*, aliquoted, and stored frozen at −70°C until titration via standard plaque assay on Vero cells. Plaque assays were performed in triplicate for each keratocyte homogenate and averaged to obtain final titer. Murine keratocyte strains were paired and assays were done simultaneously in order to eliminate inter-test variation. Data was analyzed using two-tailed student t-test comparing viral production for each mouse strain.

Statistical Analyses

Kaplan-Meier survival curves were constructed for each congenic murine strain and for each HSV-1 challenge dose, plotting observation day against the incidence of stromal keratitis.[10] Mean time to development of disease and percent resistance to HSK were calculated. Breslow test statistics were employed to analyze the significance of the individual Kaplan-Meier survival curves generated.[11]

In order to evaluate murine strain-pair differences in development of HSK, we used Cox's proportional hazards model.[12] This model is a survival analysis with covariates and generates disease ratio coefficients analogous to logistic regression models. Relative risk of developing HSK can be calculated for each strain and analyzed for significance.

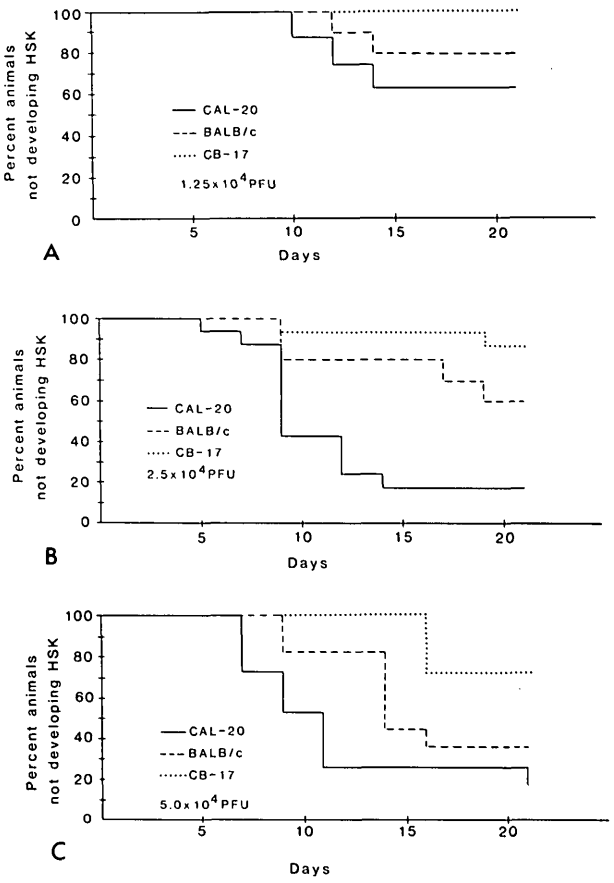

Fig. 1. Kaplan-Meier survival curves plotting resistance from keratitis against observation interval in days following corneal inoculation with (A) 1.25×10^4 PFU HSV-1 (B) 2.5×10^4 PFU HSV-1 and (C) 5.0×10^4 PFU HSV-1.

Results

Clinical Disease

The degree of keratopathy developing in these three Igh-1 congenic strains after HSV corneal inoculation followed a remarkably consistent pattern and was observed with each of the three HSV inocula employed. Once stromal keratitis developed, it typically progressed to severe 4+ HSK. The Kaplan-Meier survival curves for each HSV-1 inoculum employed are shown in Figure 1. The survival curves plot the percent of the animals not exhibiting stromal keratitis against the observation intervals in days for each mouse strain. While survival curves at the lower HSV-1 inoculum (Fig. 1A) demonstrated differences between the congenic strains, this difference was not significant. Murine strain differences were demonstrable at the higher HSV-1 challenges (Fig. 1B,C). At the 2.5×10^4 PFU inoculum, there was a statistically significant difference between survival curves (Breslow = $P < 0.00001$). The mean times for the development of HSK in the C.AL-20, BALB/cByJ and C.B-17 mice were 11.6, 18.0 and 20.2 days respec-

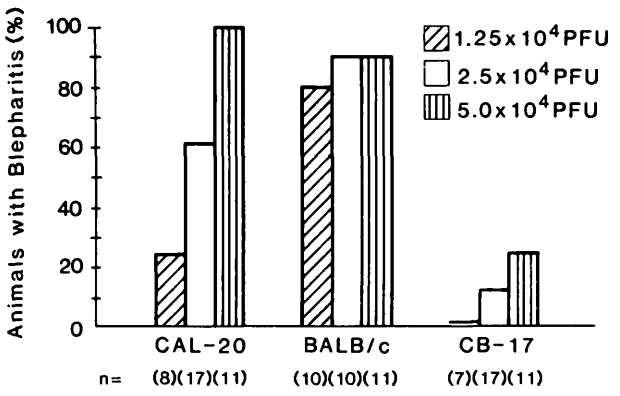

Fig. 2. Percent of animals developing blepharitis 14 days following HSV-1 corneal challenge (2.5 × 10^4 PFU HSV-1).

tively and the cumulative percentages of animals not developing clinical disease were 18%, 60% and 88% respectively. Kaplan-Meier survival curves for the 5.0 × 10^4 PFU dose generated significantly different responses between the congenic mice challenged (Breslow = $P < 0.003$). Eighty-two percent of the C.AL-20 mice, 64% of the BALB/cByJ mice and only 27% of the C.B-17 mice developed stromal disease following corneal HSV-1 challenge at this dose.

Survival analysis with covariates (Cox proportional hazards model) demonstrated a highly significant relationship between the three congenic mouse strains across all HSV-1 challenge doses employed. C.B-17 mice are 10.3 times less likely to develop HSK than C.AL-20 ($P < 0.00001$). BALB/cByJ mice were intermediate, being 3.7 times more likely to develop disease than C.B-17 ($P < 0.003$) but 2.8 times less likely to develop HSK than C.AL-20 mice ($P < 0.003$) following corneal inoculation.

The blepharitis pattern was similar to the corneal responses in the C.AL-20 and C.B-17 mice (Fig. 2). BALB/cByJ mice consistently developed a more severe blepharitis, an exception to the usual HSV-1 dose-response and a notable difference from the typical intermediate degree of pathology noted in the cornea compared to C.AL-20 and C.B-17 mice. Corneal epithelial disease paralleled stromal keratitis in both severity and frequency in the congenic mice.

To determine whether Igh-1 linked resistance is dominant, F1 progeny from C.AL-20 × C.B-17 matings were challenged with 2.5 × 10^4 PFU KOS strain HSV-1. The resulting keratitis pattern was similar to the resistant C.B-17 parent with none of the offspring (n = 9) developing stromal keratitis following corneal inoculation.

Table 1. Congenic murine keratocyte permissivity to HSV-1 strain KOS

Mouse strain	*Multiplicity of infection*	*Virus production* (PFU/10^5 Cells)*	*P-value†*
C.AL/20	5	11.6 ± 2.9 (n = 5)	NS
BALB/c	5	9.6 ± 3.0 (n = 5)	NS
C.B-17	5	11.4 ± 2.7 (n = 5)	NS

* Infected keratocyte monolayers were individually harvested and assayed simultaneously for viral production after a 24-hr incubation.
† Two-tailed student t-test.

Ganglionic Latency

The number and percent of latently infected trigeminal ganglia after in vivo corneal challenge with HSV-1 were not statistically different between the congenic mice. In C.AL-20 mice, 23/27 (85%), BALB/cByJ, 20/22 (77%) and C.B-17, 25/30 (83%) trigeminal ganglia were latently infected. Data from the cocultivation of ganglia coupled with keratitis data from individual mice indicate that ganglionic latency may develop in certain animals even when clinical keratitis was not observed.

Keratocyte Permissivity

HSV-1 replication was not significantly different in C.AL-20, BALB/cByJ or C.B-17 keratocytes with titers of 11.6 ± 2.9 PFU, 9.6 ± 3.0 PFU and 11.4 ± 2.7 PFU per 1 × 10^5 cells respectively following infection with HSV-1, MOI = 5 (Table 1).

Discussion

The host response to HSV-1 infection is determined by a complex interaction between virus strain and host genetics involving both viral replication kinetics and the immune system. Eradication of the replicating virus is an important aim in an immunocompetent host, but the immune effector mechanisms must be regulated in such a way that tissue architecture and physiology is not destroyed in the process. This caveat is well illustrated in ocular HSV-1 infections where corneal inflammation must be moderated to assure adequate transparency. In certain individuals, the host inflammatory and immune responses to HSV-1 are capable of clearing the virus without producing clinical disease while in other individuals the same system mediates a severe necrotizing stromal keratitis.[13] Genetic factors that influence the generation of severe HSK and predispose an individual to corneal blindness are not well defined. The development of a reproducible animal model of HSK which allows the study of host responses in genetically defined inbred and congenic murine strains is of considerable importance. In this communication we present such a model, employing three Igh-1 disparate congenic BALB/c mice to demonstrate a highly significant relationship between HSK and gene products encoded by loci closely linked to the Igh-1 locus on chromosome 12 and unrelated to the establishment of HSV-1 ganglionic

latency or keratocyte permissivity to HSV-1 replication.

The Igh-1 influence on HSK is evident in the survival curves and is highly significant with Breslow test statistics. Results of a Cox regression analysis further demonstrate the different response patterns in the Igh-1 disparate murine strains and generate HSK resistance ratios for each mouse pair. C.AL-20 mice with Igh-1^d phenotype develop a necrotizing stromal keratitis ten times more frequently compared to C.B-17 mice with Igh-1^b phenotype ($P < 0.00001$). Furthermore, Igh-1^a appears to be a true intermediate-response allotype, with BALB/cByJ mice developing a moderate clinical and histologic disease. This clinical disease pattern is demonstrated consistently across the three viral challenge doses and supports the relative HSK resistance scheme where the resistance of Igh-1^b phenotype is greater than Igh-1^a, which in turn is greater than Igh-1^d. These same gene products, however, do not appear to control the establishment of a functional trigeminal ganglionic latency. It would appear that the mechanisms responsible for the regulation of corneal disease do not significantly influence the pathways effecting ganglionic colonization and latent HSV-1 infection.

It is interesting to note that although in vivo resistance to intraperitoneal, cutaneous or corneal HSV-1 infection follows a dominant inheritance pattern in F1 murine progeny, in vitro host cell permissivity data prove an inverse inheritance.[1,2,5,14,15] Fibroblasts from HSV-1 in vivo-resistant F1 progeny dominantly inherit the high intracellular viral replication kinetics of the susceptible parent strain. Similarly, splenocytes and peritoneal macrophages from F1 progeny of HSV-1 resistant and susceptible mice demonstrate viral permissivity patterns associated with the susceptible parent despite an in vivo HSV-1 resistance.[16,17] It would appear that in vitro host cell permissivity differences play only a minor role in resistance to HSV-1 in vivo and that other genetically dominant effector mechanisms modulate the development of clinical disease.

Keratocytes from HSV-1 susceptible A/J (Igh-1^e) mice support a two- to four-fold, significantly higher viral replication in vitro than do cells from HSV-1 resistant C57BL/6J (Igh-1^b) mice for both KOS and MP stains HSV-1 (unpublished data). These data are in agreement with previous investigations documenting augmented viral replication in keratocytes from HSV-1 moderately susceptible BALB/c mice as compared to cultured C57BL/6J cell lines.[13] One-step viral growth differences between *inbred* mice have been proposed to play a role in resistance to HSV-1 corneal infection and in the generation of severe HSK.[6,13] Multiple HSV-1 replication cycles in vivo would generate higher viral titers in susceptible keratocyte populations and would result in higher antigen deposition in the corneal tissue. Such antigen-challenge disparity between strains could in fact lead to differential corneal immunopathology and would need to be considered in interpretations of inbred murine HSV-1 susceptibility patterns. Extrapolation from keratocyte permissivity studies in inbred mouse strains would suggest that the disease patterns observed in our congenic Igh-1 disparate mice could result from distinct, nonimmune, viral replication differences. Our data presented herein, however, establish that while there is a significant difference in HSV replication between keratocytes from inbred mice, viral replication and permissivity are equivalent in cultured keratocytes from C.AL-20 (Igh-1^d), BALB/cByJ (Igh-1^a) and C.B-17 (Igh-1^b) congenic mice. Therefore, gene products encoded by the Igh-1 locus do not govern intracellular HSV-1 replication in keratocytes. Other genes or gene products distinct from Igh-1 appear to be involved in the observed replication differences between A/J, C57BL/6J and BALB/c inbred murine keratocytes.

Igh-1 regulation of HSK supports a role for immunologic mechanisms in the generation of disease. Igh-1 encoded products are known to control both the constant and variable regions of immunoglobulin heavy chains and therefore may influence HSK via B cell modulation. Furthermore, studies concerning the immune response to other antigens have linked Igh-1 encoded structures to T cell immunomodulation.[18,19] Together, these data suggest that Igh-1 regulation of HSK may occur through an immune effector pathway controlling either B cell or T lymphocyte functions following exposure to HSV-1 antigen in the cornea. Additional studies are required to further define the relationship between the determinants encoded by the Igh-1 linked gene and host response to herpes simplex viral challenge in this mouse model. We propose that recognition of this Igh-1 linked immunogenetic regulation will result in further knowledge of the complex interaction between host and HSV-1, ultimately directing clinical management via appropriate immunomodulatory agents and therapies.

Key words: Igh-1 locus, immunogenetics, mouse, herpes simplex virus (HSV), cornea, infection

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