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Enhanced resistance to herpes simplex virus type 1 infection in transgenic mice expressing a soluble form of herpesvirus entry mediator

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Abstract

Herpesvirus entry mediator (HVEM) is a member of the tumor necrosis factor (TNF) receptor family used as a cellular receptor by virion glycoprotein D (gD) of herpes simplex virus (HSV). Both human and mouse forms of HVEM can mediate entry of HSV-1 but have no entry activity for pseudorabies virus (PRV). To assess the antiviral potential of HVEM in vivo, three transgenic mouse lines expressing a soluble form of HVEM (HVEMIg) consisting of an extracellular domain of murine HVEM and the Fc portion of human IgG1 were generated. All of the transgenic mouse lines showed marked resistance to HSV-1 infection when the mice were challenged intraperitoneally with HSV-1, but not to PRV infection. The present results demonstrate that HVEMIg is able to exert a significant antiviral effect against HSV-1 infection in vivo.

Keywords: Herpesvirus entry mediator; HveA; HVEM; HSV-1; Transgenic mouse

Introduction

Herpesviruses have been classified into three subfamilies on the basis of biological characteristics and genomic analysis. Members of the alphaherpesvirus subfamily including human herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), porcine pseudorabies virus (PRV), and bovine herpesvirus 1 (BHV-1) as representative members are neurotropic, have a short replicative cycle, and have a broad host range. Infection in the natural host is characterized by lesions in the epidermis, usually on mucosal surface, with spread of virus to the nervous system and establishment of latent infections in neurons.

Binding of alphaherpesviruses to cells occurs primarily through an interaction of virion glycoproteins (gC and/or gB) with cell surface heparan sulfate (Herold et al., 1994; Mettenleiter et al., 1990; Shieh et al., 1992; WuDunn and Spear, 1989), whereas fusion between the virion envelope and cell membrane requires the glycoproteins gB, gD, gH, and gL (Cai et al., 1988; Forrester et al., 1992; Ligas and Johnson, 1988; Roop et al., 1993). The interaction between HSV envelope gD and a specific cellular receptor is required for virus entry into mammalian cells (Campadelli-Fiume et al., 2000; Spear et al., 2000). To date, five human alphaherpesvirus receptors have been identified: herpesvirus entry mediator (HVEM, also known as HveA and TNFRSF14), a TNF receptor-related protein (Montgomery et al., 1996); three immunoglobulin superfamily members: HveB (PRR2, nectin-2), HveC (PRR1, nectin-1), and HveD (PVR, CD155); and 3-*O*-sulfated heparan sulfate (Cocchi et al., 1998; Geraghty et al., 1998; Shukla et al., 1999; Warner et al., 1998). HVEM was identified as a gD receptor for entry of HSV-1 and HSV-2 (Montgomery et al., 1996; Nicola et al., 1998; Whitbeck et al., 1997).

HVEM is expressed in lymphoid cells but also in other cell types (Kwon et al., 1997; Montgomery et al., 1996). Mice encode a related protein that is only 45% identical to

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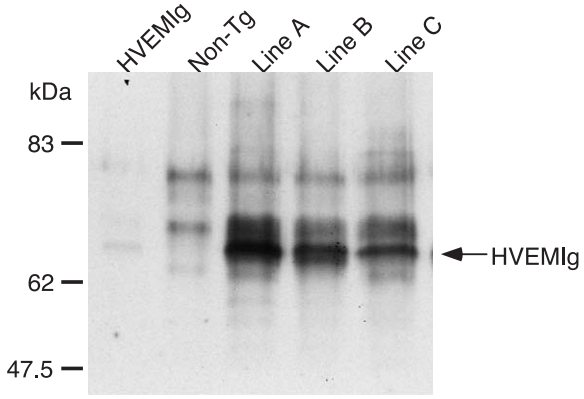

Fig. 1. Western blot analysis of tail extracts from the transgenic and non-transgenic mice. The positive control (HVEMIg) is the purified HVEMIg from the supernatant of COS7 cells infected with AxHVEMIg. The positions of molecular size markers are shown, along with the expected position of HVEMIg.

the human form (Hsu et al., 1997). Both human and mouse forms of HVEM can mediate entry of HSV-1 and HSV-2 but have no entry activity for PRV and BHV-1 (Montgomery et al., 1996; Spear et al., 2000). Natural ligands for HVEM are members of the TNF family including lymphotoxin-α and LIGHT (Mauri et al., 1998). Both can also serve as ligands for other members of the TNF receptor family. The cytoplasmic tail of HVEM can transmit signals through TNF receptor-associated factors (TRAFs) resulting, for example, in activation of NF-κB and AP1 (Hsu et al., 1997; Marsters et al., 1997). LIGHT can stimulate the proliferation of T cells, presumably through interactions with HVEM (Harrop et al., 1998a; Kwon et al., 1997). Deletion of the cytoplasmic tail from HVEM to remove all TRAF-binding sequences has no effect on the ability of HVEM to mediate HSV entry (Montgomery et al., 1996). Thus, signal transduction seems not to be required for viral entry, although signal transduction might occur when HSV enters a cell via full-length HVEM and thus influence downstream events in viral replication or virus–cell interaction.

We have set our sights on inhibition of viral entry for the approach to germ-line transformation in animals to confer resistance to virus infection. It has been shown that secreted forms of HVEM bind to the virus by an interaction with HSV-1 gD and block infection by HSV-1 (Geraghty et al., 1998; Nicola et al., 1998; Whitbeck et al., 1997). In the present study, transgenic mouse lines expressing a soluble form of HVEM were established and resistance to HSV-1 infection in the transgenic mice was then examined to determine the antiviral potential of the soluble form of HVEM in vivo.

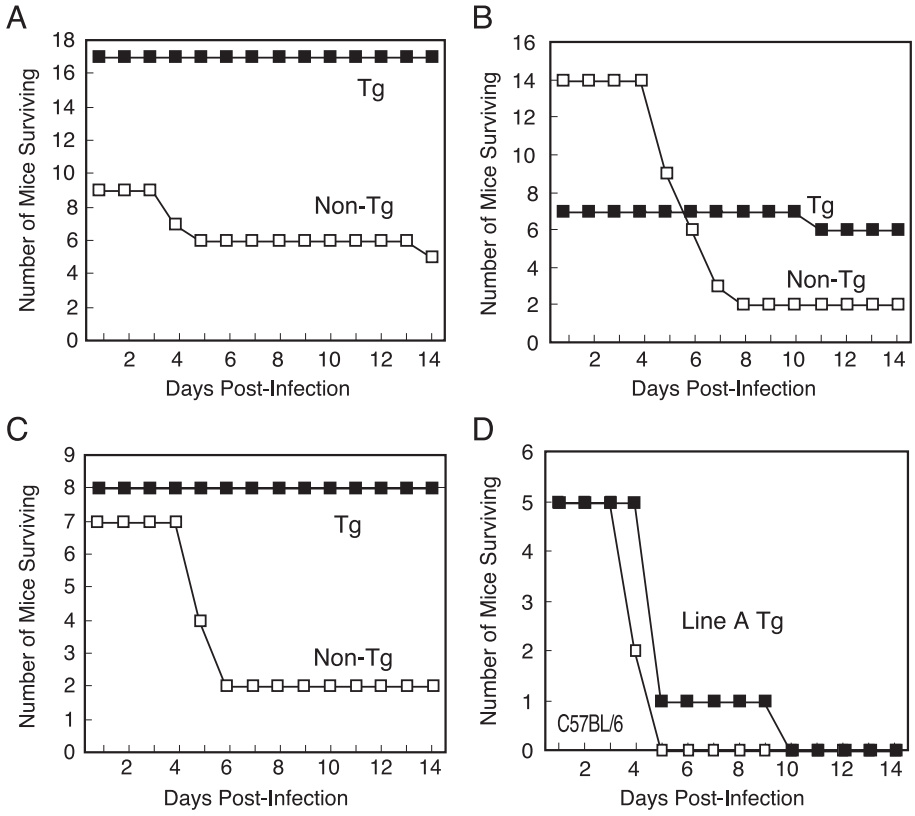

Fig. 2. Resistance to HSV-1 infection in the transgenic mouse lines A (A), B (B), and C (C). The transgenic (closed squares) and non-transgenic (open squares) mice were challenged intravenously with HSV-1. In a control experiment, transgenic mice of the line A and C57BL/6 mice were challenged with PRV (D). The mice were observed for their survival times, which are recorded daily for 14 successive days after the challenge.

Results

Characterization of transgenic mice

To assess the antiviral potential of HVEM in vivo, we generated three transgenic mouse lines expressing a soluble form of HVEM (HVEMIg) consisting of an extracellular domain of murine HVEM and the Fc portion of human IgG1. In these transgenic mice, HVEMIg was expressed under the control of the CAG promoter, which is expected to allow ubiquitous expression in all cell types (Niwa et al., 1991). To prepare HVEMIg, an adenovirus vector containing the HVEMIg gene (AxHVEMIg) was constructed. HVEMIg was purified from the supernatant of AxHVEMIg-infected COS7 cells by Protein A-Sepharose. A specific band whose molecular size is comparable to that of purified HVEMIg was detected by Western blot analysis using rabbit anti-HVEMIg antibody in each transgenic mouse line (Fig. 1). HVEMIg concentrations in serum samples of the transgenic mouse line A were 17.3–29.6 μg/ml, those of the lines B and C were 4.4–8.5 μg/ml and 14.2–24.3 μg/ml, respectively.

Resistance to HSV-1 infection in the transgenic mice

To find out whether the transgenic mice expressing HVEMIg were protected from HSV-1 infection, 10 LD_{50} of HSV-1 determined on C57BL/6 mice was intravenously inoculated into the transgenic mice and their non-transgenic littermates. Because these transgenic founders were obtained from B6SJLF1 (C57BL/6 X SJL) mouse embryos and the offspring of a heterozygous of the F3 progeny and C57BL/6 parents were used for the experimental infection, the 50% lethal doses of HSV-1 were determined on C57BL/6 and SJL mice. The LD_{50} of HSV-1 on C57BL/6 and SJL mice were 10^8 and 10^7 PFU, respectively. Based on the results, 10^9 PFU of HSV-1 was inoculated into the mice. The survival data (Fig. 2) demonstrate that all of the transgenic mouse lines showed remarkable resistance to HSV-1 infection. All of transgenic mice of the line A completely survived the viral inoculation and remained healthy for several months following this trial. In contrast, seven of nine littermates of the line A developed symptoms such as paralysis or died within 14 days after HSV-1 infection. Only one transgenic mouse of the line B, which is low expression line, died after the infection and other six transgenic mice survived. In contrast, 13 of 14 littermates of the line B developed the symptoms or died within 14 days. In the line C, all of the transgenic mice completely survived the viral inoculation, and six of seven littermates of the line C developed the symptoms or died. To confirm whether signs of disease such as paralysis were due to encephalitis caused by HSV-1 infection, histopathological analyses were performed. As shown in Fig. 3A, brains obtained from the non-transgenic mice infected with HSV-1 exhibited typical encephalitic lesions such as perivascular cellular infiltration, gliosis, and neuronal degeneration. In contract, such severe lesions were not observed in the

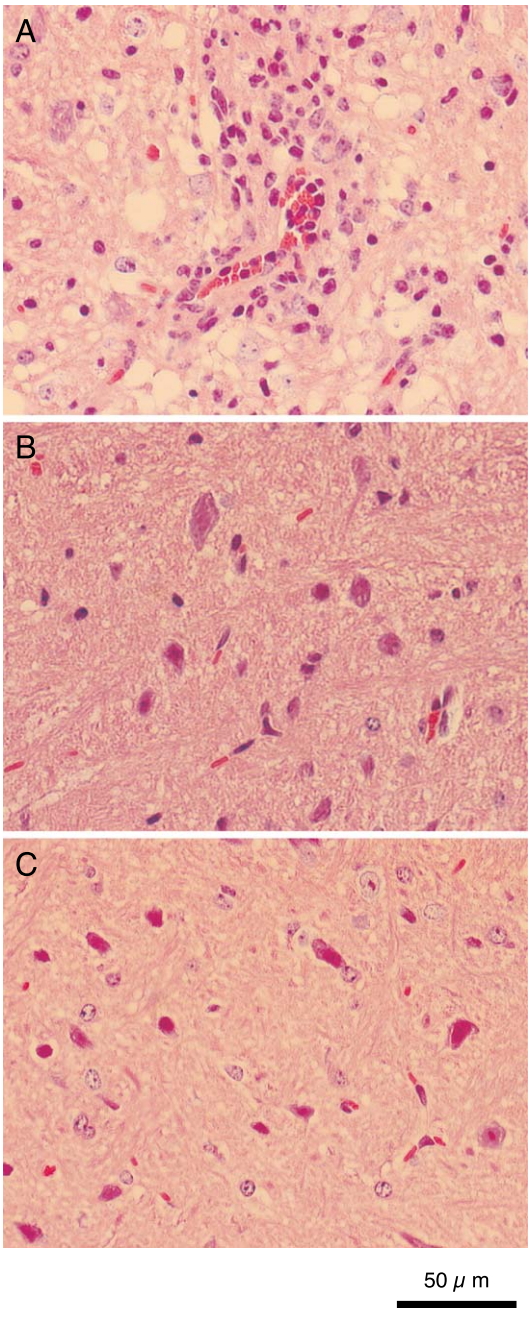

Fig. 3. Histopathological analyses of mouse brains infected with HSV-1. The non-transgenic mouse brain (A) and the transgenic mouse brain (B) infected with HSV-1, and a mock-infected control mouse brain (C) in sagittal sections were stained with hematoxylin and eosin.

transgenic mice infected with HSV-1 (Fig. 3B) as in mock-infected control mice (Fig. 3C). During latent infection, HSV-1 LATs are detected in the trigeminal ganglia (Spivak and Fraser, 1987; Stevens and Cook, 1971). RT-PCR analysis was performed to detect LATs in the trigeminal ganglia of survived mice. LATs expression was observed in all of the recovered littermates that developed the symptoms and only one transgenic mouse of the line B that exhibited no symptom, but not in all of other transgenic mice and the survived littermates without symptom. In a control experi-

ment, to find out whether the transgenic mice expressing HVEMIg were protected from PRV infection, 10 LD_{50} (viral dose determined on C57BL/6 mice; 250 PFU) of PRV was intravenously inoculated into transgenic mice of the line A and C57BL/6 mice because HVEM dose not act as a gD receptor for PRV. All of the transgenic and control mice died within 10 days after PRV infection (Fig. 2D). The mice used in these challenge experiments were the offspring of heterozygous transgenic and non-transgenic parents. The use of non-transgenic littermates as negative controls in these experiments minimizes the possible contributions of variables not associated with the presence of the transgene. It is, therefore, considered that the only difference between experimental and control animals is the presence of the functional HVEMIg gene in the transgenic animals which showed marked resistance to HSV-1 infection.

Because herpesviruses usually enter the body via infection of mucosal epithelium, RT-PCR analysis was performed to detect LATs in the trigeminal ganglia of mice after intranasal inoculation with 10^4 PFU of HSV-1. As shown in Fig. 4, LATs expression was not observed in 17 of 22 transgenic mice. In contrast, eight of nine littermates were infected with HSV-1. In the virus inoculation with 10^6 PFU of HSV-1, however, all of the transgenic mice were infected with HSV-1 in addition to their littermates (data not shown). Unfortunately, even in the highest dose tested, no symptom was developed by the intranasal inoculation. These results indicate that expression of HVEMIg protects from HSV-1 infection via mucosal inoculation and the protection depends on the virus dose.

Inhibition of HSV-1 infection by HVEMIg in sera of the transgenic mice

To examine the antiviral activity in sera of the transgenic mice, a virus plaque reduction assay was performed. It was found that sera of the transgenic mouse line C protected Vero cells against HSV-1 infection but not PRV infection. In contrast, sera of non-transgenic mouse failed to show any protection against both virus infections. This antiviral activity was neutralized by preincubation with the rabbit anti-HVEMIg antibody. In addition, level of the antiviral activity was correlated with the concentrations of HVEMIg in serum. The results of plaque assays were presented in Table 1. In the assays using sera of other transgenic mice, similar inhibition was observed. These results demonstrated that HVEMIg in sera of the transgenic mice is able to inhibit HSV-1 infection.

Table 1
Inhibition of HSV-1 infection by HVEMIg in sera of the transgenic mice

Serum (HVEMIg μg/ml)[a]	Plaque number[b]	
	HSV-1	PRV
C2 (14.2)	0	58.0 ± 8.8
(1.42)	0	NT
(0.14)	1.7 ± 1.6	NT
(0.01)	34.7 ± 16.2	NT
C2 (0.14) + anti-HVEMIg	30.3 ± 6.9	NT
Control	44.0 ± 0	53.7 ± 2.9

[a] Determined by competitive ELISA.
[b] The value is the average of triplicated infections and standard deviation is shown.

Inhibition of HSV-1 infection in cultured embryonic fibroblasts from the transgenic mice

To assess whether the in vivo resistance to HSV-1 infection in the transgenic mice was paralleled by resistance of their isolated cells, we tested embryonic fibroblasts for resistance to HSV-1 infection in culture. Transgenic and non-transgenic embryo fibroblasts were prepared from embryos of the heterozygous transgenic (the line B) and non-transgenic parents. HVEMIg expression in the transgenic fibroblasts was confirmed by Western blot analysis, whereas HVEMIg was not detected in the non-transgenic fibroblasts (Fig. 5A). The transgenic and non-transgenic fibroblasts were infected with 50 PFU of HSV-1. In the transgenic fibroblasts, number of plaques was extensively suppressed as compared with the non-transgenic fibroblasts 5 days postinfection (Fig. 5B). In contrast, there was no difference between the transgenic and non-transgenic fibroblasts infected with PRV (Fig. 5B). To monitor virus growth in the transgenic fibroblasts, virus infectivities in the media

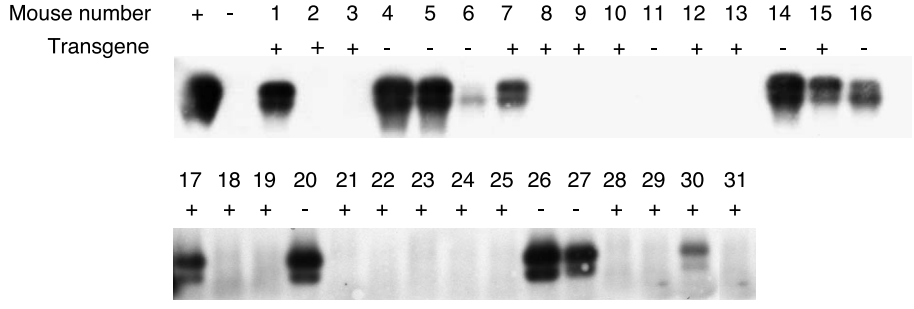

Fig. 4. Expression of LATs in the trigeminal ganglia of mice after intranasal inoculation of HSV-1. LATs expression in the trigeminal ganglia of the transgenic mice of line C and their littermates was examined by RT-PCR 2–3 weeks after the virus inoculation. Southern blot analysis of the RT-PCR products was performed using the LATs-specific probes. The positive control (+) is a product synthesized from the total RNA of PRV-infected Vero cells and the negative control (−) is a product from that of mock-infected Vero cells.

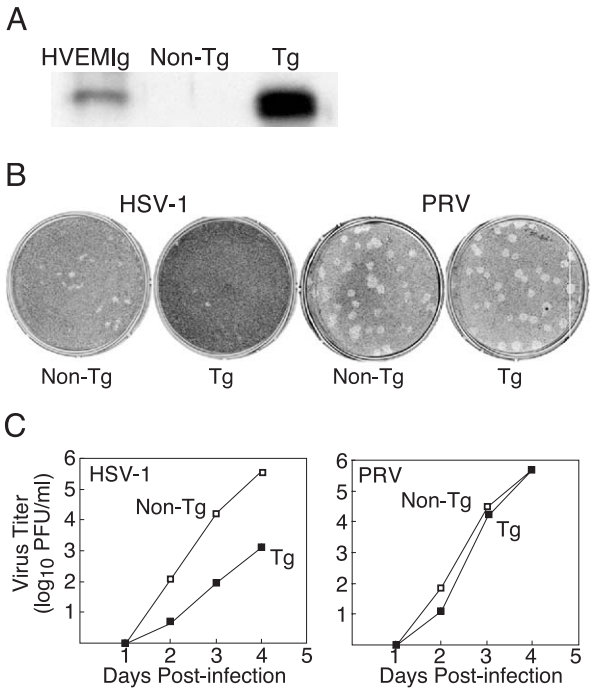

Fig. 5. Resistance to HSV-1 infection in embryonic fibroblasts prepared from the transgenic mice. (A) Western blot analysis of cell extracts from the transgenic (Tg) and non-transgenic (Non-Tg) fibroblasts. The positive control (HVEMIg) is the purified HVEMIg from the supernatant of COS7 cells infected with AXHVEMIg. (B) Plaque assays of HSV-1 and PRV infection on the embryonic fibroblasts. Plaque assays were performed as described in Materials and methods. (C) HSV-1 and PRV growth curves in embryonic fibroblasts prepared from the transgenic (closed squares) and non-transgenic (open squares) mice.

were titrated (Fig. 5C). In the transgenic fibroblasts, virus yield was extensively suppressed (100-fold less in virus titer than the non-transgenic fibroblasts 2 and 3 days postinfection). The transgenic fibroblasts were susceptible to infection with PRV as the non-transgenic fibroblasts (Fig. 5C). These results indicate that HVEMIg expressed from the transgenic fibroblasts specifically inhibit HSV-1 infection to the fibroblasts.

Discussion

Infections of HSV-1 are highly prevalent among humans. Pathologic manifestations vary from mucocutaneous lesions of the mouth, face, eyes, or genitals, to involvement of central nervous system, resulting sometimes in encephalitis (Whitley, 1996). In the present study, transgenic mice expressing HVEMIg showed significant resistance to HSV-1 infection when the mice were challenged intraperitoneally with HSV-1. This resistance was much more striking than that observed in HSV-1-resistant transgenic mouse lines expressing a mutant allele (X25) of ICP4 (Smith and DeLuca, 1992). The demonstration that HVEMIg secreted into sera of the transgenic mice is sufficient to compete with the virus infectivity may provide a basis for the future development of a novel class of anti-HSV agent designed specifically to block HSV-1 infection.

Consistent with the earlier studies (Geraghty et al., 1998; Nicola et al., 1998; Whitbeck et al., 1997), a secreted form of HVEM, HVEMIg, in sera of the transgenic mice blocked HSV-1 infection. In addition, the transgenic embryonic fibroblasts showed marked resistance to HSV-1 infection. These results indicate that secreted HVEMIg may have important roles on the resistance to HSV-1 infection in the transgenic mice. Almost complete protection from intravenous infection may suggest a possibility that the primary effect of the fusion protein is to bind to virus in serum and promote its clearance by macrophage via the Fc portion of human IgG. This effect of the fusion may enhance the resistance to the virus infection in vivo. LATs expression in the trigeminal ganglia was not detected in the survived transgenic mice except for only one transgenic mouse of the line B, indicating that protection against intravenous infection with HSV-1 in the transgenic mice was almost complete. Likely, the transgenic mouse of the line B expressing LATs was infected with HSV-1. This finding suggests that HVEMIg may suppress manifestation of the disease. Only two transgenic mice of the line B, the lowest expression line, were infected and only one mouse died, indicating that the level of resistance to HSV-1 infection may be correlated with the concentrations of HVEMIg in serum.

Protection from intranasal HSV-1 infection was proved by RT-PCR analysis for detection of LATs expression in the trigeminal ganglia. In 75% of transgenic mice inoculated intranasally with HSV-1, LATs expression was not detected. In contrast, more than 90% of non-transgenic littermates allowed to the virus infection (Fig. 4). However, transgenic mice inoculated with higher doses of HSV-1 could not prevent the viral infection. These findings indicate that transgenic mice expressing HVEMIg may be able to prevent HSV-1 entry into the body via mucosal epithelium, in addition to intravenous rote.

HSV infections in mice are influenced by the host genetic background, and multiple dominantly inherited genes are involved (Lopez, 1975). Although these results have been known for a long period of time, no particular host gene that can control the outcome of experimental HSV-1 infection in mice has so far been identified. The effectiveness of resistance mechanisms is not only age dependent, but also regulated by genetic factors that are generally virus specific. In adult mice, the resistance to HSV-1 infection is regulated by non-*H-2* genes (Lopez, 1975, 1980). For HSV-1, C57BL/6 mice are genetically resistant (Kumel et al., 1982; Zwatzky et al., 1982). Because our transgenic mice may contain the HSV-1-resistant background, we cannot completely exclude a possibility that the genetic control of resistance to HSV-1 infection may be involved in the observed resistance in the transgenic mice. However, susceptibility of their non-transgenic littermates to HSV-1 infection was demonstrated in the present study. Furthermore, all of the transgenic mouse lines showed marked resistance to HSV-1 infection. It is, therefore,

considered that resistance to HSV-1 infection in the transgenic mice was conferred by the manipulation of only one gene.

The major concern in the transgenic mice is adverse side effects caused by the expressed HVEMIg. However, we observed no gross abnormality while breeding the transgenic mouse lines. The transgenic mice developed normally and there was no difference in the body weights of the transgenic mice and their non-transgenic littermates. HVEM is a member of the tumor necrosis factor (TNF) receptor family and a receptor for lymphotoxin-α (LTα) and LIGHT (Mauri et al., 1998) and plays an important role on the immune systems. A possibility is that HVEMIg affects the host immune responses against the insulting agents because HVEMIg can also block the binding between HVEM and LIGHT, resulting in the inhibition of T cell activation (Harrop et al., 1998b). Although the possibility could not be eliminated in the transgenic mice used in the present study, significant difference in the immune responses was not observed, at least when the transgenic and non-transgenic mice were immunized with ovalbumin (data not shown). It seems likely that fatal disturbances of the immune responses do not occur in the transgenic mouse lines.

The animals described herein expressing HVEMIg constitutively demonstrated significant resistance to HSV-1 challenge. It is noteworthy that protection against HSV-1 entry in the sites of primary infection was observed in the transgenic mice via intranasal inoculation of HSV-1. The potential that suppresses HSV-1 replication in the embryonic fibroblasts may support this finding. Several possible effects of HVEMIg on the suppression of virus replication in the primary fibroblasts are considered. First, HVEMIg inhibits initial infection to the fibroblasts. Second, HVEMIg also inhibits secondary infections of the fibroblasts with free virus released in the first round infection. Third, HVEMIg inhibits secondary infection in the fibroblasts mediated by cell to cell spread. These possible effects of HVEMIg on the nasal mucosa could be considered, resulting in protection from HSV-1 infection.

Adverse side effects such as toxicity and infertility were characteristic of herpesvirus-resistant transgenic mice (Ono et al., 1999; Smith and DeLuca, 1992). However, no side effect is observed in the three transgenic mouse lines. These transgenic mouse lines showed normal growth rates and they are fertile. Therefore, it may be possible to use similar kind of transgenes encoding soluble forms of the receptors for an approach to germ-line transformation in agricultural livestock to confer resistance to animal herpesvirus infection such as pseudorabies virus.

Materials and methods

Virus and cells

Herpes simplex virus type 1 (HSV-1) strain VR-3 was kindly provided by T. Suzutani (Asahikawa Medical College, Asahikawa, Japan). Vero cells were a generous gift from M. Peeples (Rush University, Chicago, IL). Vero cells, grown in RPMI1640 medium supplemented with 5% fetal bovine serum, 0.03% L-glutamine, 100U/ml penicillin, and 0.1mg/ml streptomycin were used for propagation of HSV-1. Pseudorabies virus (PRV) strain YS-81 and a cellular clone of the porcine kidney cell line (CPK) were kindly provided by M. Shimizu (National Institute of Animal Health, Tsukuba, Japan). CPK cells, grown in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum, 0.03% L-glutamine, 0.03% tryptose phosphate broth, nonessential amino acids (Flow Laboratories, Costa Mesa, CA), vitamins (Flow Laboratories), 100 U/ml penicillin, and 0.1 mg/ml streptomycin, were used for propagation of PRV. Vero cells were also used for plaque assay. COS7 cells, grown in Dulbecco's modified Eagle medium (DMEM; Sigma Co., St. Louis, MO) supplemented with 10% fetal bovine serum, 0.03% L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin were used for preparation of HVEMIg.

Preparation of HVEMIg

To prepare a soluble form of HVEM (HVEMIg), an adenovirus vector containing a chimeric gene that encodes a fusion protein consisting of an extracellular domain of murine HVEM and the Fc portion of human IgG1 was constructed. The extracellular domain of HVEM cDNA was amplified by using RT-PCR, in which an mRNA isolated from Con A-stimulated splenocytes obtained from BALB/c mouse was used as a template. The PCR primers used to amplify the cDNA were 5′-TAACTCGAGCTCTTGGCCTGAAGTTTC-3′ and 5′-TTAAGGATCCGAGGAGCAGGTGGTGTCT-3′. The cDNA was inserted into the *Xho*I and *Bam*HI sites of a plasmid carrying IgG1-Fc DNA (Nakagawa et al., 1998), and verified the sequence. Then, the plasmid was digested with *Xho*I and *Xba*I to obtain the HVEMIg DNA fragment. The fragment was blunt-ended and was inserted into a *Swa*I site of pAxCAwt cosmid vector (Takara, Kyoto, Japan) to prepare the recombinant adenovirus (AxHVEMIg). The recombinant adenovirus was prepared using Adenovirus Expression Vector Kit (Takara) according to the protocol supplied by manufacturer. HVEMIg was purified from a supernatant of COS7 cells infected with AxHVEMIg. Briefly, semi-confluent COS7 cells were washed and infected with AxHVEMIg. After 4 days culture, the supernatant was recovered for the purification of HVEMIg. HVEMIg was purified from the supernatant by Protein A-Sepharose 4FF (Amersham Pharmacia Biotech, Buckinghamshire, England) affinity chromatography (Takiguchi et al., 1999).

Transgenic mice

The *Pme*I–*Sal*I transgene fragments containing the CAG promoter (cytomegalovirus IE enhancer and chicken β-actin promoter), the HVEMIg gene, and the rabbit β-globin polyA

signal were isolated and purified. The DNA fragments were microinjected into C57BL/6 X SJL F2 hybrid mouse eggs to create transgenic mice in Chrysalis DNX Transgenic Sciences Corporation (Princeton, NJ). Transgenic founders were identified by PCR using genomic DNA isolated from mouse tail (Hogan et al., 1986) and the specific primers (5′-GCCTCTGCTAACCATGTTCATGCCT-3′ and 5′-TGTGCTGCAAGCATGTGGAACAGTG-3′), which resulted in the amplified fragment of 573 bp. The transgenic founders were crossed with C57BL/6 mice until we obtained the third (F3) generation. Each heterozygous of the F3 progeny crossed with C57BL/6 mice and their offspring were used for the experimental infection. All mice were maintained in the animal facility at our institute and treated according to the Laboratory Animal Control Guidelines of our institute, which basically conformed to those of the National Institutes of Health-American Association of Laboratory Animal Control.

Analysis of the transgene expression

To detect HVEMIg in sera of the transgenic mice, polyclonal antibodies against HVEMIg were raised in NZW rabbit immunized with the purified HVEMIg. Whole serum was collected and used as anti-HVEMIg antibody. To measure HVEMIg concentrations in sera of the transgenic mice, a competitive ELISA system was established. The 96-well plate was coated with 5 μg/ml HVEMIg then treated with 1% BSA solution to block nonspecific protein binding. Both of serum sample and 10^6-fold diluted anti-HVEMIg antibody were added into the well simultaneously and incubated for 1 h. After washing the wells, 10^4-fold diluted peroxidase-labeled goat anti-rabbit IgG (BIO-RAD Laboratories, Hercules, CA) was added and then visualized antibody binding by using *o*-phenylenediamine as a substrate. Serially diluted HVEMIg were used for making a standard curve.

Western blotting was performed by the method of Towbin et al. (1979). Ten microliters of each extract from tails of the transgenic mice was separated by 12% SDS-polyacrylamide gel electrophoresis. The separated proteins were then electrophoretically transferred to Immobilon transfer membrane (Millipore, Bedford, MA). The sheet was treated sequentially with a Block Ace (Snow Brand Milk Products, Sapporo, Japan), 500-fold diluted anti-HVEMIg antibody, and finally with peroxidase-labeled goat anti-rabbit IgG. The antigen was detected using Renaissance Reagent (DuPont NEN Research Products, Boston, MA) as a substrate.

Virus infection in mice

Groups of adult (7 weeks old) C57BL/6 and SJL mice were inoculated intravenously with 100 μl aliquots of serial 10-fold dilutions of HSV-1 strain VR-3 and the survival rate was assessed daily for 14 days. The 50% lethal doses (LD_{50}) of the virus strain in each mouse strain were determined graphically. Transgenic and non-transgenic offspring of the F3 progeny were used when they were 7 weeks old. To verify the transgene expression, sera of the mice were collected before the experimental infection. The mice were infected intravenously with 100 μl of phosphate-buffered saline (PBS) containing 10 LD_{50} (viral dose determined on C57BL/6 mice) of HSV-1. Survival of mice and signs of disease were recorded for 14 days. The transgene expression was verified by the competitive ELISA after the experimental infection was finished. Experimental infection with PRV was also performed using the transgenic and C57BL/6 mice as described above.

To test for protection of the mice after inoculation of mucosa, the mice were infected intranasally with 5 μl of PBS containing 10^4 PFU of HSV-1 under anesthesia. Trigeminal ganglia were obtained 14 days after the virus inoculation to detect LATs expression by RT-PCR.

Histopathological procedure

Mice were killed by decapitation after the termination of the experiments at day 14. Tissue samples from decapitated animals were excised and immersion-fixed in Bouin's solution for 24 h at room temperature, embedded in paraffin wax, and cut into 5-μm-thick slices with a microtome. Sections were stained with hematoxylin and eosin.

Detection of LATs in trigeminal ganglia by RT-PCR

Survived mice were killed by decapitation and trigeminal ganglia were immediately removed and frozen in liquid nitrogen. Total cellular RNA was isolated from the trigeminal ganglia using TriZOL reagent (Invitrogen, Grand Island, NY). For RT-PCR analysis, 1 μg of total RNA was digested with RNase-free DNaseI (Invitrogen) to remove any contaminating genomic DNA. The cDNA was synthesized from the DNaseI-treated total RNA by MMLV reverse transcriptase (Invitrogen) using ICP0-3′ (Iwai et al., 1991) as a LAT-specific primer. The PCR reaction was carried out as described by Halford et al. (1996). Control samples without the reverse transcriptase reaction were amplified in parallel to confirm the absence of genomic DNA contamination. The PCR products were fractionated on 1.5% agarose gel and transferred to Hybond N^+ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) by capillary blotting. Digoxigenin (DIG)-labeled DNA probes for detection of LATs were derived from pSLAT2 (Lachmann et al., 1996) using the specific primers LAT-a and LAT-b (Lynas et al., 1989) and a PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). Hybridization and detection of LATs were performed as described previously (Ono et al., 1999).

Competition of the virus infectivity with HVEMIg

Vero cells were plated at a subconfluent density on 35-mm dishes and cultured for 24 h. The following day, 5 μl of serum sample was incubated with 50 PFU of HSV-1 or PRV

in 1 μl at 37 °C for 1 h. After Vero cells were washed twice with DMEM, each sample containing viruses was absorbed to Vero cells at 37 °C for 1 h. Viruses were removed and then overlaid with DMEM containing 0.5% agarose (Sigma). Plaques were counted 3–4 days after infection.

Virus infection in mouse embryonic fibroblasts

Transgenic and non-transgenic mice (embryonic day 14) were killed by decapitation, and the embryonic fibroblasts were isolated by dicing the mouse embryos, stirring the pieces for 30 min at room temperature in PBS supplemented with 0.25% trypsin and then plating out the resulting single cell suspension. The cells were grown in DMEM supplemented with 10% fetal bovine serum and the above antibiotics. The cells were passed no more than twice before use. To detect HVEMIg, the mouse embryonic fibroblasts were plated at a subconfluent density on 60-mm dishes. The following day, the cells were lysed in the sample buffer. Aliquots of the extracts were applied onto 12% SDS-polyacrylamide gels and electrophoresed. Western blotting was performed as described above.

For the plaque assay, the mouse embryonic fibroblasts were plated at a subconfluent density on 35-mm dishes. The following day, 50 PFU of HSV-1 or PRV was adsorbed onto the cells. After 1 h, the cells were washed twice with DMEM and then overlaid with agar described above. The cells were stained 3–4 days after infection. To monitor virus growth in the mouse embryonic fibroblasts, the cells were plated at a subconfluent density on 60-mm dishes. The following day, 0.01 PFU/cell of HSV-1 or PRV was adsorbed onto the cells. After 1 h, the cells were washed twice with DMEM and then maintained in 5 ml of DMEM. Supernatant was removed at 24-h intervals. Virus titers of supernatant samples were determined on Vero cell monolayers.

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