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Transgenic Mouse Lines That Are Immunologically Tolerant and Nontolerant to Herpes Simplex Virus Glycoprotein D

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A construct containing the gene for glycoprotein D of herpes simplex virus (HSV-gD), under the control of the simian virus 40 early promoter, was microinjected into single-cell embryos, and four transgenic mouse lines were established. Three were homozygous (lines 75, 111, and 113) and one was hemizygous (line 108) for the HSV-gD gene. Examination of sera revealed that only one of the lines (line 75) spontaneously produced antibody to HSV-gD. Immunization of the other three lines with vaccinia virus-HSV-gD showed that one of them (line 113) responded by making antibody to HSV-gD, whereas the other two (lines 108 and 111) appeared to be immunologically tolerant. Evidence that tolerance was not absolute was obtained by immunization with infectious HSV, which resulted in an antibody response to HSV-gD in some of the animals from line 111. Examination of organs for HSV-gD mRNA revealed transcripts in the tolerant line (line 108) and in the partially tolerant line (line 111), but not in the nontolerant line (line 113), suggesting that the development of immunological tolerance requires active expression of the HSV-gD gene.

Ordinarily, the immune system of an organism does not respond to self-antigens. Immunological unresponsiveness or tolerance is thought to develop during early embryogenesis and involves both B and T lymphocytes (3, 10, 17). The mechanisms resulting in the production of tolerance and the factors that may result in its breakdown and cause autoimmune disease are not well understood. By use of transgenic technology, it is possible to introduce foreign genes into mice and determine whether the gene products are viewed by the host's immune system as self or nonself (2, 6, 11, 12, 14, 16, 25).

Glycoprotein D of herpes simplex virus (HSV-gD) is a powerful immunogen (4, 9, 15, 20). It is expressed on both the surface of the virus and the surface of infected cells. Mature animals exposed to infectious HSV or immunized with HSV-gD develop neutralizing antibodies that protect against infection. Although the evidence is far from definitive, the immune response to certain viruses, through mechanisms such as molecular mimicry, is thought to be capable under some circumstances of triggering autoantibodies and perhaps even autoimmune disease (13, 21).

In the present study, transgenic mice carrying the HSV-gD gene were produced and the immune response to HSV-gD was evaluated in terms of antibody production, immunological tolerance, and autoimmunity.

MATERIALS AND METHODS

Preparation of the DNA construct. Cloning and expression of the HSV-gD gene under the control of the simian virus 40 (SV40) early promoter-enhancer (pSVgD) were described previously (18). A linear 7.0-kb recombinant molecule (see Fig. 1A) was produced by digestion with restriction enzymes *Bam*HI and *Eco*RI and subsequently purified for microinjection.

Production of transgenic mice. Single-cell embryos derived from inbred FVB/N mice (National Institutes of Health breeding stock, Harlan Sprague-Dawley) were microinjected with pSVgD according to standard techniques (7) and transferred into pseudopregnant FVB/N recipients. Founder animals were tested for the presence of pSVgD sequences by slot blot and Southern blot analysis. Animals positive for the transgene were bred to establish several lineages of transgenic mice maintained in the FVB/N strain.

RNA isolation and analysis (Northern blots). Total RNA was isolated from various organs by the RNAzol method (Cinna/Biotecx, Friendswood, Tex.). Northern blots were prepared by transfer of RNA to nylon membranes (Nytran; Schleicher and Schuell, Keene, N.H.) and hybridized to the entire ^{32}P-labeled pSVgD plasmid or to an 800-bp *Pvu*II-*Apa*I fragment spanning the 5′ region of the HSV-gD gene. Filters were washed twice at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then twice at 55°C with 0.1× SSC.

Immunization with HSV-gD. Six-month-old animals were inoculated intradermally with 10^8 PFU of recombinant vaccinia virus-HSV type 1 (HSV-1)-gD (4). Blood was drawn from the retro-orbital plexus at various times postvaccination to test the sera for the presence of antibodies to HSV-gD.

HSV-1 challenge. Animals 5 to 7 months old were inoculated intraperitoneally with 4×10^3 tissue culture infectious doses of HSV-1 strain McIntyre. The challenge dose was selected on the basis of preliminary experiments to examine the susceptibility of FVB/N mice to HSV-1. Serum samples from surviving animals were analyzed for the presence of anti-HSV-gD and anti-HSV antibodies.

Immunofluorescence assay for anti-HSV-gD antibodies. Serum samples were tested for the presence of anti-HSV-gD antibodies by indirect immunofluorescence with a pSVgD-transfected line of NIH 3T3 cells previously shown to express surface HSV-gD (18). Cell monolayers were acetone fixed and incubated with serial dilutions of test mouse sera beginning at a 1:40 dilution. Bound antibodies were visualized with fluorescein-conjugated goat anti-mouse immunoglobulin (i.e., immunoglobulin M [IgM], IgG, and IgA) antibodies. Selected positive sera were further analyzed by

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TABLE 1. Characterization of HSV-gD-transgenic mice

Mouse line	Approx. no. of HSV-gD copies/ haploid genome	Status of line for HSV-gD
75	135	Homozygous
108	1	Hemizygous
111	1	Homozygous
113	6	Homozygous

using fluorescein-conjugated, affinity-purified goat anti-mouse IgM (μ-chain-specific) or anti-mouse IgG Fc fragment (γ-chain-specific) antibodies (Organon Teknika-Cappel, West Chester, Pa.).

HSV neutralization assay. A complement-dependent microneutralization assay was used to measure anti-HSV titers with normal rabbit serum as a source of complement (15). Twofold serial dilutions, starting at 1:20, of mouse sera were incubated with 100 PFU of HSV-1. Exponentially growing Vero cells were inoculated with the reaction mixture and examined for cytopathic effects 3 days later.

Histology. Morphological analysis was performed on 5-μm-thick paraffin sections of various tissues from pSVgD-transgenic mice for evidence of autoimmune disease or other pathological changes. Sections were stained with hematoxylin and eosin.

RESULTS

Establishment of transgenic lines. Four lines of FVB/N mice carrying the HSV-gD gene under the control of the SV40 early promoter-enhancer were derived (Table 1). Three of the four transgenic founders (lines 75, 111, and 113) produced homozygous lines in the F_2 generation. Line 108 was maintained in the hemizygous state because of a recessive lethal effect exerted by the transgene. Slot blot analysis revealed that animals from lines 108 and 111 each carried 1 copy of pSVgD per haploid genome, whereas animals from lines 75 and 113 carried 135 and 6 copies of pSVgD per haploid genome, respectively.

Southern analysis of *Hin*dIII-digested DNA (Fig. 1B) showed the presence of a single pSVgD copy only in animals from line 111. It could be deduced from the number, size, and intensity of the pSVgD-hybridizing bands that animals from line 108 carry a single copy of pSVgD slightly truncated at the 5′ end in addition to two or three short fragments of pSVgD construct integrated nearby (all the pSVgD-hybridizing bands cosegregated in all the tested animals from line 108). Further analysis (not shown) revealed that these short pSVgD fragments, but not the prominent 6.8-kb fragment, hybridized to the plasmid-SV40 probe. The *Hin*dIII restriction pattern of DNA from line 113 was consistent with the presence of several copies of pSVgD joined in a single head-to-tail array. The DNA from transgenic line 75 was not easily amenable to restriction enzyme analysis because of the very high copy number.

Antibodies to HSV-gD in sera of transgenic lines. To see whether the transgenic animals recognized the product of the HSV-gD gene as foreign, serum samples of animals bled between 1 and 21 months of age were tested for antibodies to HSV-gD. As seen in Table 2, serum samples from three transgenic lines (lines 108, 111, and 113) were found to be serologically negative for HSV-gD. However, within line 75, approximately 25% of the animals produced antibodies of the IgG isotype to HSV-gD. The antibody titer to HSV-gD

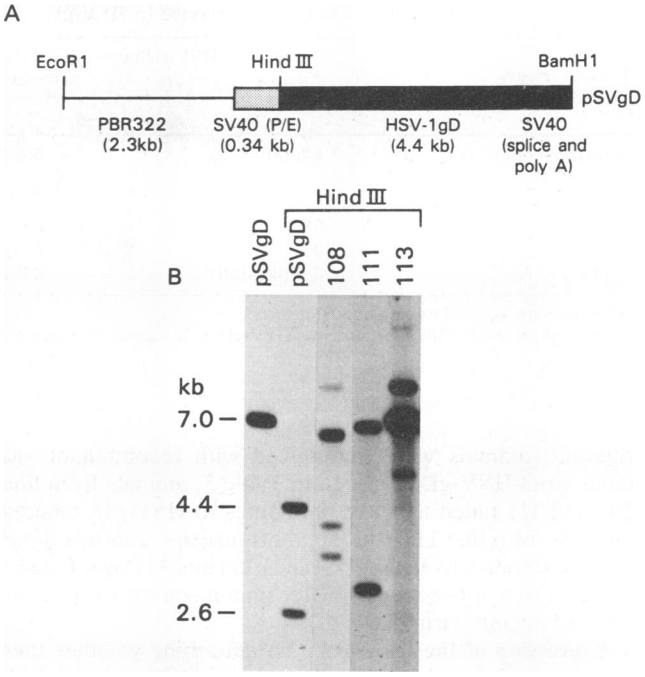

FIG. 1. Identification of transgenic mice carrying HSV-gD DNA. (A) A 4.4-kb HSV-gD cDNA (solid bar) was cloned downstream of the SV40 promoter-enhancer (stippled bar) and upstream of splice and polyadenylation sequences derived from SV40. The plasmid containing this construct (pSVgD) was restricted with *Eco*RI and *Bam*HI to produce a 7.0-kb linear recombinant molecule for microinjection. (B) Southern blot analysis of single-copy, undigested pSVgD DNA, *Hin*dIII-digested pSVgD DNA, and *Hin*dIII-digested tail DNA from transgenic lines 108, 111, and 113. *Hin*dIII cleaves the linearized pSVgD construct at a single site at the junction of the SV40 promoter-enhancer and the HSV-gD cDNA, generating a 2.6-kb 5′-end fragment and 4.4-kb 3′-end fragment.

ranged from 40 to 1,280 by immunofluorescence and from 20 to 640 by neutralization of infectious HSV (not shown). These animals appeared healthy despite the presence of antibodies to HSV-gD for many months. Histological examination of the tissues of these animals failed to reveal any evidence of autoimmunity or other pathological changes.

Antibody response to HSV-gD in transgenic mice immunized with vaccinia virus-HSV-gD. To determine whether transgenic animals that did not produce antibodies to HSV-gD (lines 108, 111, and 113) were immunologically

TABLE 2. Antibody to HSV-gD in sera of transgenic mice[a]

Mouse type and line	No. of anti-HSV-gD-positive mice/no. tested	Titer[b]
Nontransgenic	0/62	
Transgenic		
75	39/161	40–1,280
108	0/22	
111	0/20	
113	0/12	

[a] Sera were assayed by immunofluorescence.
[b] Reciprocals of highest serial twofold dilution, beginning at 1:40, showing positive immunofluorescence. Data are expressed as a range of positive titers.

TABLE 3. Antibody response to HSV-gD in transgenic mice immunized with vaccinia virus-HSV-gD

Mouse type and line	No. anti-HSV-gD positive/no. tested (titer)[a] the following no. of wks after primary immunization:			No. anti-HSV-gD positive/no. tested (titer) after booster[b]
	3	5	9	
Nontransgenic	6/6 (80–320)	5/6 (40–80)	4/6 (40–80)	6/6 (80–160)
Transgenic				
108	0/6	0/6	0/6	0/6
111	0/6	0/6	0/6	0/6
113	4/4 (40–160)	4/4 (40–320)	3/4 (160–320)	4/4 (40–640)

[a] Sera were assayed by immunofluorescence.
[b] Reimmunization with vaccinia virus-HSV-gD at 10 weeks after primary immunization. Sera were tested 2 weeks later.

tolerant, animals were immunized with recombinant vaccinia virus-HSV-gD. As seen in Table 3, animals from lines 108 and 111 failed to make antibodies to HSV-gD, whereas animals from line 113 and the nontransgenic controls developed antibodies to HSV-gD. Lines 108 and 111 also failed to respond to a subsequent booster immunization with recombinant vaccinia virus-HSV-gD.

Expression of the transgene. To determine whether there was any relationship between the tolerant state and the expression of the gene, tissue from tolerant (lines 108 and 111) and nontolerant (line 113) transgenic lines were examined for the presence of HSV-gD transcripts. HSV-gD transcripts were detected to various degrees in the spleen, thymus, lymph nodes, kidney, brain, adrenal glands, heart, lungs, skeletal muscles, ovary, and uterus but not in the bone marrow, liver, pancreas, or salivary gland of line 108. A similar organ distribution of transcripts was found for line 111, except that no detectable HSV-gD transcripts were found in muscle or lungs. Representative Northern blots, showing the various levels of expression, are seen in Fig. 2. The HSV-gD message was detected as early as day 8 of gestation in line 111 and was very high for placenta. In contrast to the results for the tolerant lines, the HSV-gD message was not detected in any organs from mature animals of the nontolerant line (113), nor was it present in fetuses at 8, 12, or 16 days of gestation.

Antibody response to HSV-gD after challenge with infectious HSV-1. To determine whether tolerance could be overcome by presenting HSV-gD in a different context, animals from lines 108 and 111 were challenged with the entire infectious HSV. As seen in Table 4, animals from line 108 did not make antibody to HSV-gD. However, about one-half of the animals from line 111 did mount a response to HSV-gD at 12 and 18 weeks after challenge. In contrast to animals from lines 108 and 111, the nontransgenic controls made a vigorous immune response to HSV-gD at 6 weeks after challenge. In a second experiment, similar results were obtained, except that at 18 weeks the only survivor of HSV challenge from line 108 and four survivors from line 111 were weakly positive compared with nontransgenic controls (data not shown). Antibodies were of the IgG isotype.

Table 4 also shows that by 6 weeks all of the mice from line 111 and by 18 weeks most of the mice from line 108 developed neutralizing antibody. The absence of antibody to HSV-gD in animals from line 108 argues that the neutralizing antibody is directed to other antigenic components (i.e., non-HSV-gD) on the surface of the infectious virus to which the transgenic animals are not tolerant.

DISCUSSION

In this report, we described the production and properties of four transgenic mouse lines that carry the gene for HSV-gD. Two of the transgenic lines, 108 and 111, did not spontaneously produce antibody to HSV-gD and were nonresponsive to immunization with recombinant vaccinia virus-HSV-gD. Nonresponsiveness of this type is considered a condition of immunological tolerance (23). Evidence that the tolerance was not absolute, however, was demonstrated by immunization with infectious HSV, which resulted in the production of low levels of antibody to HSV-gD in some of the animals. For a number of viral antigens, complete tolerance has been difficult to achieve (19).

Any of several different mechanisms, including clonal deletion, clonal anergy, enhanced suppressor cell activity, and anti-idiotypic antibody production, may underlie tolerance. In fact, studies with transgenic mice have shown that

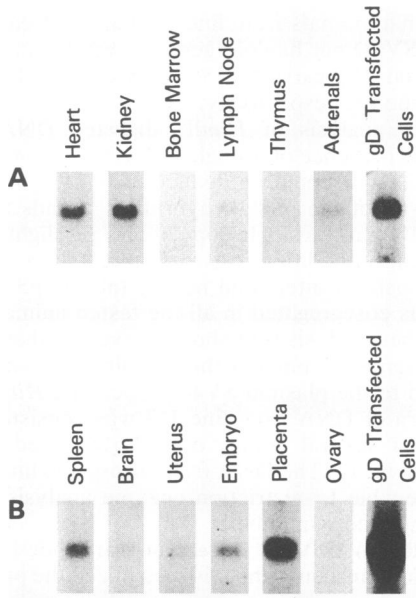

FIG. 2. Northern blots of RNA isolated from transgenic line 111, showing various levels of HSV-gD message in tissues. NIH 3T3 cells transfected with pSVgD DNA served as positive controls. Equal amounts of total cellular RNA were loaded per lane as judged by ethidium bromide staining. Blots were probed with an approximately 800-bp *Pvu*II-*Apa*I fragment spanning the 5′ region of the pSVgD gene (A) and with the entire ^{32}P-labeled pSVgD plasmid (B).

TABLE 4. Challenge of transgenic mice with infectious HSV-1

Mouse type and line	No. anti-HSV-gD positive/no. tested (titer)[a] the following no. of wks after immunization:			No. positive by neutralization/no. tested (titer)[b] the following no. of wks after immunization:		
	6	12	18	6	12	18
Nontransgenic	4/4 (80–640)	4/4 (160–1,280)	4/4 (640–1,280)	4/4 (40–160)	4/4 (80–160)	4/4 (80–320)
Transgenic						
108	0/4	0/4	0/4	2/4 (40–80)	2/4 (80–160)	3/4 (80–640)
111	0/4	2/4 (80)	2/4 (40)	4/4 (80–320)	4/4 (40–160)	4/4 (80–640)

[a] Sera were assayed by immunofluorescence. Titers represent reciprocals of the highest serial twofold dilution, beginning at 1:40, showing positive immunofluorescence. Ranges of titers are in parentheses.

[b] Sera assayed by complement-dependent microneutralization. Titers represent reciprocals of the highest serial twofold dilution, beginning at 1:20, giving neutralization. Ranges of titers are in parentheses.

both clonal deletion and clonal anergy of self-reactive lymphocytes can result in a state of nonresponsiveness for B and T cells (2, 6, 14, 16). Findings not dissimilar to those described here for HSV-gD have been recently reported for mice transgenic for the glycoprotein G (gG) of vesicular stomatitis virus (25). It is of particular interest that in both of these studies, abrogation of B-cell tolerance followed challenge with the respective infectious virus but not with HSV-gD or vesicular stomatitis virus gG expressed by a vaccinia virus recombinant. How the viral antigen is presented appears to be pivotal in breaking tolerance.

The immune response to HSV-gD is thought to contribute to the pathogenesis of HSV lesions (9). However, in the present studies, animals from line 75, which spontaneously produced antibodies to HSV-gD, did not develop autoimmune disease. Similarly, animals that developed antibodies to HSV-gD after immunization with infectious HSV (e.g., line 111) failed to show signs of autoimmune disease. One explanation is that HSV-gD is not expressed in appreciable quantities on the surface of cells of our transgenic animals. In addition, many autoimmune diseases are associated with specific major histocompatibility complex haplotypes (22, 24) and FVB/N mice may not be of the susceptible type. A higher level of expression of the HSV-gD transgene in mice with a different major histocompatibility complex background may be required to produce autoimmune disease.

In the present study, the two transgenic lines that showed tolerance (lines 108 and 111) expressed HSV-gD transcripts not only in adulthood but also during gestation. Transgenic lines which silently carry the gene but do not express the HSV-gD message, such as line 113, would not be expected to spontaneously make antibody to HSV-gD or develop tolerance. Our findings support the argument that tolerance is an active process which requires expression of the gene product at critical stages in embryonic development. Similarly, it has been proposed that different levels of tolerance may be achieved and maintained as a result of differential exposure to tolerogenic stimuli during development (1, 5). Thus, the time and degree of expression of integrated foreign viral genes, as well as aberrations in the expression of the host's own genes, may prove to be important factors in both the development of the immune response to endogenous viruses (8) and the triggering of autoimmune disorders.

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